RATIONALLY DESIGNED ANTIBODIES

Related Applications

This application claims priority to U.S. Provisional Patent Application No. 60/251,448 filed December 5, 2000, and to U.S. Provisional Patent Application No. 60/288,889 filed May 4, 2001, and to U.S. Provisional Patent Application No.

5   60/294,068 filed May 29, 2001. The disclosures of these three provisional applications are incorporated herein in their entirety by this reference.

TECHNICAL FIELD

The present invention relates to antibody molecules and biologically active

10   peptides as diagnostic and therapeutic reagents.

BACKGROUND OF RELATED ART

Antibodies are produced by B lymphocytes and defend against infection. Antibodies are produced in millions of forms, each with a different amino acid

15   sequence. Antibody molecules are composed of two identical light chains and two identical heavy chains. When digested by the enzyme papain, two identical Fab fragments are produced along with one Fc fragment. When digested with the enzyme pepsin one $F(ab')_2$ fragment is produced. Light and heavy chains consist of constant and variable regions. Within the variable regions are hypervariable regions (aka

20   complementarity determining regions (CDRs)) which form the antigen binding site. The remaining parts of the variable regions are referred to as framework regions.

Important biological functions, such as receptor binding, activation and enzymatic activity, are often attributable to discrete regions of larger protein molecules, comprising a limited number of amino acid residues.   Peptides displaying binding,

25   activation or enzymatic activity have also been discovered by screening libraries of peptides generated by the random linking of amino acid residues. These peptides may not correspond to a linear arrangement of amino acids in a larger protein molecule exhibiting similar biological activity and are referred to as discontinuous

peptide epitopes or mimotopes. Certain peptide mimetics have been described and cloned. See, e.g., U.S. Pat. No. 6,083,913 (thrombopoietin (TPO) mimetic), U.S. Pat. No. 5,835,382 (erythropoietin (EPO) mimetic), U.S. Pat. No. 5,830,851 (EPO mimetic) and Wrighton et al, Science, (1996) 273:458-63. Peptide epitopes and mimotopes due to their small size are potentially advantageous over large protein molecules for use as therapeutic reagents. However, the results with these peptides as therapeutics may often be unsatisfactory. One drawback to the use of peptides as therapeutic reagents is that they are generally unstable *in vivo*, i.e., their clearance rates from serum may be quite rapid. In addition, it is difficult to predict the activity, therapeutic or otherwise, of a peptide if it is fused into a larger molecule since conformational changes and other molecular forces may interfere with or totally negate the activity of the peptide. Attempts have been made to introduce certain polypeptides into CDR regions of antibodies. See, e.g., PCT Appln. WO 94/18221. However, as mentioned previously, due to conformational changes which may be caused by surrounding amino acids, the biological activity of active polypeptides may be diminished or negated. Therefore, it is an object herein to provide rationally designed antibodies or fragments thereof which include biologically active peptides for use as diagnostic and therapeutic reagents.

SUMMARY

Provided herein are biologically active recombinant antibodies and fragments thereof that mimic the activity of biologically active peptides, methods of making such antibodies and methods for their use in therapy and diagnosis. These antibodies and fragments thereof do not suffer from some of the disadvantages of isolated peptides, as antibodies naturally have long serum half-lives and are highly specific in binding their target. It has surprisingly been found that incorporation of particular amino acids surrounding a target peptide that has been combined into an antibody molecule actually increases the biological activity of the peptide.

Immunoglobulins or fragments thereof have a peptide of interest inserted into a complementarity determining region (CDR) of an antibody molecule. The antibody molecule serves as a scaffold for presentation of the peptide and confers upon the peptide enhanced stability. The peptide optionally replaces all the amino acids of a

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CDR region, or may be added to an existing CDR, whereby the original antigen specificity is disrupted, wherein the CDR region is defined by either of the two accepted schemes (See, Kabat et al., Sequences of Proteins of Immunologics Interest, 5th ed (1991), NIH Publication 91-3242 and Chothia et al. J.Mol. Bio (1992)

5   (227)776-98.) Furthermore, additional amino acids may be randomly introduced which flank the peptide and allow for the screening of optimum peptide presentation in the antibody framework. It has been surprisingly found that in certain cases a proline flanking the peptide provides an increase in biological activity.

In particular embodiments an immunoglobulin molecule or fragment has amino

10   acids residues corresponding to one complementarity determining region (CDR) replaced with amino acid residues comprising a biologically active hemopoietic or thrombopoietic peptide. In another particular embodiment, amino acid residues corresponding to at least two complementarity determining regions (CDRs) are each replaced by amino acid residues comprising such a biologically active peptide. In a

15   single immunoglobulin molecule or fragment thereof, one or more complementarity determining regions can be replaced with a peptide; for example, CDR3 of a heavy chain, CDR3 of a light chain, CDR3 of both a heavy and light chain, CDR2 and CDR3 of a heavy chain, or CDR2 and CDR3 of a light chain. Other combinations of replaced CDR regions are possible, including the replacement of CDR1. In addition, instead of

20   replacement of a CDR, one could add the peptide to a native CDR without actual replacement of amino acid residues while still disrupting the original antigen specificity.

Thus, in one aspect, a biologically active peptide is provided with enhanced activity by adding a proline to its carboxy terminus to form a proline-extended biologically active peptide which is used to replace or add to at least a portion of at

25   least one CDR region in an immunoglobulin molecule or fragment thereof. In another aspect, an immunoglobulin molecule or fragment thereof is provided which has either a TPO mimetic peptide or EPO mimetic peptide as a replacement for at least one native CDR region. In this aspect, the TPO mimetic peptide or EPO mimetic peptides may optionally be proline-extended as described herein.

30   In further particular embodiments the immunoglobulin molecule or fragment thereof is an Fab, a ScFv, a heavy chain variable region, a light chain or a full IgG

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molecule. The immunoglobulin molecule or fragment thereof can also have a dimerization domain, so as to enable immunoglobulin molecules which have only one CDR replaced with a peptide to dimerize and thus activate receptors that require dimerization for activation.

5   In certain embodiments, the biologically active peptide can be a linear peptide epitope or a discontinuous peptide epitope. Furthermore, the biologically active peptide, when substituted for a CDR region, can have in addition to proline, one, two or more additional flanking amino acid residues proximate to the amino and/or the carboxyl termini of the peptide, which are positioned between the peptide and
10   immunoglobulin framework region residues (i.e., at what was the junction between a CDR and the adjoining framework). The flanking amino acid residues are not typically present in the active peptide. If preferred flanking amino acid residues are already known, the flanking amino acid residues are encoded by codons which designate those specific amino acid residues. However, by initially utilizing codons, such as
15   NNK, NNY, NNR, NNS and the like, which designate multiple amino acid residues, a collection of peptides that differ from one another merely by the flanking residues is generated. The flanking amino acid residues may determine the presentation of the peptide in the immunoglobulin molecule or fragment thereof and thus may influence the binding and/or biological activity exhibited by the peptide. This random collection
20   of flanking amino acids allows for the selection of the best context to display the peptide sequence within the antibody framework that results in specific binding to the target molecule and the exhibition of optimal biological activity. Screening of libraries of immunoglobulins having a common peptide but different flanking amino acid residues can be carried out using binding, growth and activation assays known by
25   those skilled in the art and as described herein.

The peptide replacing the amino acid residues comprising a CDR can be any peptide which specifically binds a target molecule and whose utility could be altered by incorporation in an antibody framework. The peptide could also exhibit a specific activity (e.g., agonist, antagonist, enzymatic, etc.). In a particular embodiment the
30   peptide is an agonist or an antagonist for a cell surface receptor. For example, the cell surface receptor can be for a cytokine, a growth factor, or a growth inhibitor.

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In particularly useful embodiments, replacement of at least a portion of a CDR with a peptide provides an antibody that acts as an agonist. The peptide used to replace at least a portion of a CDR may itself have agonist properties. Alternatively, the peptide (although specifically binding to a receptor) may not exhibit agonist activity. Rather, agonist activity might be exhibited only when the peptide is substituted for at least a portion of a CDR and is thus present in the engineered antibody. In such embodiments, the presence or absence of proline flanking the peptide is not critical, but can, in some instances, be preferred.

Thus, in one aspect the present disclosure provides for an agonist antibody comprising an antibody framework engineered to contain at least one biologically active peptide inserted at, or in place of at least a portion of, one or more CDRs. The biologically active peptide may or may not exhibit agonist activity prior to insertion into the antibody framework. In certain embodiments the antibody framework is engineered to contain two peptides capable of dimerizing with each other.

In yet another aspect, the present disclosure provides for an immunoglobulin molecule or fragment thereof comprising a region where amino acid residues corresponding to at least a portion of a complementary determining region (CDR) are replaced with a biologically active peptide, whereby the immunoglobulin molecule or fragment thereof exhibits agonist activity. The biologically active peptide may or may not exhibit agonist activity prior to insertion into the antibody framework. In particularly useful embodiments the immunoglobulin molecule or fragment thereof exhibits c-mpl agonist activity.

In yet another aspect, the present disclosure provides for an immunoglobulin molecule or fragment thereof comprising a biologically active peptide inserted at a complementary determining region (CDR), whereby the immunoglobulin molecule or fragment thereof exhibits agonist activity.

In yet another aspect, the present disclosure provides for an immunoglobulin molecule or fragment thereof comprising a region where amino acid residues corresponding to at least a portion of a complementary determining region (CDR) are replaced with a biologically active peptide, whereby the immunoglobulin molecule or fragment thereof exhibits c-mpl agonist activity.

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In further particular embodiments, the peptide replacing the amino acids of a CDR is an agonist TPO mimetic peptide. One such agonist peptide has at least the sequence IEGPTLRQWLAARA (SEQ. ID. NO. 1). Other sequences are possible for TPO agonist mimetic peptides, which can be found using binding, growth and activation assays known by those skilled in the art and as described herein. Agonist TPO mimetic peptides when positioned in CDR regions can have one or more additional amino acid residues at the amino and/or carboxyl termini of the peptide which become covalently bonded to immunoglobulin framework residues. One such TPO mimetic peptide has an additional proline residue added to the carboxyl terminus; IEGPTLRQWLAARAP (SEQ. ID. NO: 2). Other immunoglobulin molecules or fragments thereof have a CDR region replaced by the TPO mimetic peptides comprising the amino acid sequence of SEQ. ID. NOs: 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, and 49 (see Fig. 5).

Another biologically active peptide that can replace the amino acid residues of a CDR is an agonist EPO mimetic peptide. One such EPO agonist peptide has as its amino acid sequence DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 3). Other amino acid sequences are possible for EPO agonist mimetic peptides, which can be found using binding, growth and activation assays known by those skilled in the art and as described herein. Agonist EPO mimetic peptides when located in CDR regions can also have one or more additional amino acid residues at the amino and/or carboxyl termini of the peptide which become covalently bonded to immunoglobulin residues. Thus, in particular embodiments provided herein are immunoglobulin molecules (IgG) or fragments (e.g., Fab, scFv, heavy or light chains) that have a CDR region replaced with a TPO or EPO mimetic peptide. For example, the TPO peptide can include at least the sequence IEGPTLRQWLAARA (SEQ. ID. NO:1) and may further optionally have an additional proline at the immediate downstream position. The EPO mimetic encompasses at least the sequence DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 3). Likewise, it may optionally have an additional proline at the immediate downstream position.

Any immunoglobulin molecule (antibody) or fragment thereof could potentially provide the framework and have a CDR replaced with a peptide according to the

present disclosure. For therapeutic or *in vivo* diagnostic use it is preferable that the antibody is of human origin or humanized, such as an anti-tetanus toxoid immunoglobulin. Furthermore, independent of or in conjunction with the presence of additional flanking amino acids bound to the peptide, one or more amino acid residues

5   in other regions of the immununoglobulin, other CDR region(s) and/or framework regions, can be altered to modify the binding, activity and/or expression displayed by the peptide in the context of the immunoglobulin molecule.

It is contemplated that after construction of biologically active recombinant antibodies and/or fragments thereof, such recombinants can be subjected to

10   randomization methods known in the art to introduce mutations at one or more points in the sequence to alter the biological activity of the antibodies. After generation of such mutants using randomization methods such as those described herein, the resulting recombinants may be assayed for activity using binding, growth, expression and activation assays.

15   Further provided are nucleic acid molecules encoding immunoglobulin molecules or fragments thereof which have the amino acids of one or more CDR regions replaced by a biologically active peptide. These nucleic acid molecules can be present in an expression vector, which can be introduced (transfected) into a recombinant host cell for expression of these molecules. Also provided are methods

20   of producing an immunoglobulin molecule or fragment thereof containing a biologically active peptide, comprising culturing a recombinant host cell under conditions such that the nucleic acid contained within the cell is expressed.

Also provided are compositions, comprising an immunoglobulin molecule or fragment thereof which has amino acid residues corresponding to a CDR replaced

25   with amino acid residues comprising a TPO or EPO mimetic peptide and a pharmaceutically acceptable carrier.

Further provided are EPO mimetic peptides with additional flanking residues which are suitable for replacement of CDRs. Also provided are nucleic acid molecules encoding these peptides.

30   Further provided are methods of engineering immunoglobulin molecules or fragments thereof to exhibit an agonist activity in which a biologically active peptide

replaces at least a portion of one or more CDR regions of light and/or heavy chains. The methods encompass inserting a nucleic acid molecule encoding a biologically active peptide in place of at least a CDR region of a nucleic acid molecule encoding an immunoglobulin heavy or light chain or adding the molecule to the native CDR sequence and then expressing the nucleic acid molecule encoding the immunoglobulin heavy or light chain variable domain along with its complementary variable region domain, such that the two domains associate.

Further provided are methods of engineering immunoglobulin molecules or fragments thereof to exhibit an activity (property) of a biologically active TPO or EPO peptide in which a biologically active peptide replaces one or more CDR regions of light and/or heavy chains. The methods encompass inserting a nucleic acid molecule encoding a biologically active peptide in place of at least a portion of a CDR region of a nucleic acid molecule encoding an immunoglobulin heavy or light chain or adding the molecule to the native CDR sequence; and expressing the nucleic acid molecule encoding the immunoglobulin heavy or light chain variable domain along with its complementarity variable region domain, such that the two chains associate.

In another aspect, this disclosure provides a method for producing in a polypeptide a binding site capable of binding a preselected agent, the method including the steps of introducing a nucleotide sequence that codes for an amino acid residue sequence defining said binding site into a CDR region of a nucleic acid comprising an immunoglobulin heavy or light chain gene by amplifying the CDR region of the immunoglobulin gene, the introduced nucleotide sequence having the formula – X_a-Y-X_b wherein X is the same or different at each occurrence and represents a randomizing trinucleotide, the sum of a and b is 4 or less and Y is a nucleotide sequence that encodes a minimum recognition domain of said binding site. In particularly useful embodiments, amplification is achieved using overlap PCR, however, any known amplification technique could be employed, such as, for example, the methods disclosed in WO94/18221, the disclosure of which is incorporated herein by reference.

In yet another aspect, this disclosure provides methods for creation of a library of monoclonal antibodies that can be screened for a desired activity. These methods

of making a library include the steps of inserting a nucleic acid molecule encoding a biologically active peptide into, or in place of at least a portion of, one or more CDR regions of a nucleic acid molecule encoding an immunoglobulin heavy or light chain, providing up to a pair of randomizing trinucleotides on either side of the inserted nucleic acid molecule, and expressing a library of monoclonal antibodies. In particularly useful embodiments, a pair of randomizing trinucleotides is provided on both sides of the inserted nucleic acid molecules. The library of monoclonal antibodies thus produced can then be screened for a desired activity.

In a specific embodiment, antibodies and fragments thereof have different amino acids flanking the peptide at the amino and the carboxyl termini where the peptide becomes bound to the antibody scaffold. This, results in a population of antibody molecules or fragments thereof that may differ in the presentation of the peptide. The population is screened for those antibodies that exhibit the biological activity of the peptide. In a preferred embodiment, the amino acid immediately adjacent the peptide is a proline.

If the activity of the biologically active peptide is to activate a target molecule, this may require dimerization of two target molecules (e.g. receptors in the hematopoietic superfamilies). For dimerization to occur, two peptides must be positioned to each bind a target molecule such that the two bound target molecules can then properly associate. This can be accomplished by having two peptides present on the same antibody or fragment thereof or by causing two antibody molecules each containing one peptide to bind together. Thus, for example, a single peptide can be inserted into or substituted for at least a portion of a CDR and then expressed as an immunoglobulin or a F(ab')$_2$ fragment. As another example, two peptides can be inserted into or substituted for at least a portion of one or more CDRs and expresses as any antibody or antibody fragment.

The screening of antibodies or fragments thereof can be accomplished by panning with cells that have surface molecules to which the peptide specifically binds. Solid phase binding using purified target molecules or fragments thereof can also be used. Binding can also be carried out in solution using labeled target molecules. In

addition, antibodies or fragments thereof can be screened by the use of biological assays for agonist or antagonist activity of the peptide.

Also provided are libraries of different immunoglobulin molecules or fragments thereof wherein amino acid residues corresponding to a complementarity determining region (CDR) are replaced with amino acid residues comprising a biologically active peptide which has at least one additional amino acid residue at the amino or the carboxyl terminus and the immunoglobulin molecules or fragments thereof differ by the additional amino acid residue of the peptide.

In specific embodiments the biologically active peptide is a TPO mimetic or an EPO mimetic. The antibodies of the library are displayed on phage.

Further provided are methods of stimulating proliferation, differentiation or growth of cells, which include contacting the cells with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a biologically active peptide which binds to a receptor on the cells surface. In specific embodiments the biologically active peptide is a TPO mimetic or an EPO mimetic.

In other specific embodiments, is provided a method of stimulating proliferation, differentiation or growth of megakaryocytes by contacting megakaryocytes with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a TPO mimetic peptide. Also provided is a method of increasing platelet production, which involves contacting megakaryocytes with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDR regions replaced with a TPO mimetic peptide. Also provided is a method of stimulating megakaryocytes and/or increasing platelet production in a patient, in which an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a TPO mimetic peptide is administered to a patient in need thereof. The immunoglobulin molecule and the megagakarocytes can also be contacted in vitro and the resultant cells can be introduced into the patient. In addition, an antibody or fragment thereof having at least one TPO mimetic peptide incorporated therein can be administered to a subject who intends to donate platelets, thus increasing the capacity of a donor to generate platelets to provide a more robust source of such platelets.

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Also provided herein is a method of stimulating proliferation, differentiation or growth of hematopoietic cells, comprising contacting the cells with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a EPO mimetic peptide.

5   Also embodied herein is a method of activating a homodimeric receptor protein, by contacting the receptor with an immunoglobulin molecule or fragment thereof having a CDR region replaced with a biologically active peptide that specifically binds the receptor and which has been dimerized. In a further embodiment the receptor is a thrombopoietin receptor.

10   DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of the vector pRL4.

Figures 2A and B show the sequence of the human tetanus toxoid antibody framework, light and heavy chains, respectively.

Figure 3 is a diagram depicting the grafting of the TPO mimetic peptide
15   AF12505 into the heavy chain CDR3 region of the tetanus toxoid framework antibody. XX represents flanking random amino acids.

Figure 4 is a diagram of the construction of a peptide cloned into the heavy chain CDR3 region.

Figure 5 represents the amino acid and nucleotide sequences of clones that
20   encode TPO mimetic peptide AF1205 with different random flanking residues.

Figure 6A-C depicts the nucleic acid sequence of plasmid pRL8 (SEQ. ID. NO: 60). pRL8 is a modified version of pRL4 (pRL4 is also known as pComb 3X). The pRL4 was modified between the Spe I and neighboring Sfi I restriction sites (shown by underlining) to include a flexible linker (murine kappa hinge region) followed by a Jun
25   leucine zipper dimerization domain.

Figure 7 is a schematic depiction of a portion of the plasmid pRL8.

Figure 8 depicts the nucleic acid sequence of a portion of plasmid pRL8 (SEQ. ID. NO: 52) along with amino acid sequences corresponding to certain delineated nucleic acid sequences (SEQ. ID. NO: 53).

30   Figure 9 is a chart showing sequences of certain TPO positive clones herein.

Figure 10 is a bar graph showing activity of certain Fab clones containing 2 TPO mimetic peptides.

Figure 11 is a bar graph showing activity of certain Fab clones containing 2 or 3 TPO mimetic peptides.

Figure 12 graphically depicts the activity of Clone 59 as reflected by induction of luciferase activity.

Figure 13A depicts the amino acid sequence and nucleic acid sequence of the 5G1.1-TPO heavy chain (SEQ. ID. NOS: 67 and 68, respectively).

Figure 13B depicts the amino acid sequence and nucleic acid sequence of the 5G1.1 light chain (SEQ. ID. NOS: 69 and 70, respectively).

Figure 14 is a bar graph showing FACS analysis of cMpl receptor binding of purified 5G1.1+ TPO mimetic peptide compared to parental 5G1.1 antibody.

Figure 15 is a bar graph showing comparative activity of 5G1.1 antibody containing the TPO mimetic peptide in connection with cells transfected with a control vector containing no cMpl-R and cells transfected with a vector containing cMpl-R.

Figure 16 shows the sequence of clone 429/Xb4 (SEQ. ID. NO: 116)

Figure 17 is a flow chart showing the initial steps for making vector pRL5-Kappa.

Figure 18 is a flow chart showing additional steps for making vector pRL5-Kappa.

Figure 19 is a map of vector pRL5.

Figure 20 is a schematic of vector pRL5-Kappa.

Figure 21A-I show the nucleic acid sequence of vector pRL5-Kappa.

DETAILED DESCRIPTION

As used herein, "immunoglobulin" refers to an entire immunoglobulin molecule or molecules that contain immunologically active portions of whole immunoglobulin molecules and includes Fab, F(ab´)2, scFv, Fv, heavy chain variable regions and light chain variable regions. The terms immunoglobulin and antibody are used interchangeably herein.

Any peptide that exhibits a useful property is suitable for insertion in an antibody framework. Peptide activities and uses include, but are not limited to, binding a receptor, binding a membrane bound surface molecule, binding a ligand, binding an enzyme or structural protein, activating or inhibiting a receptor, targeted drug delivery, or any enzymatic activity. Those peptides whose utility can be increased from the enhanced stability conferred upon them when presented in the context of an immunoglobulin molecule are usually selected. It should be understood that "biological activity" as used herein includes any activity associated with a molecule having activity in a biological system, including, but not limited to, the stimulatory or inhibitory activity triggered by protein-protein interactions as well as the kinetics surrounding such interactions including the stability of a protein-porotein complex. Enhancing or increasing "biological activity" herein is meant to include an increase in overall activity or an increase in any component of overall activity. It should be understood that a peptide may exhibit one biological activity (such as, e.g., simply binding to a target) before insertion into the antibody framework, and a different or enhanced biological activity (such as, e.g., agonist activity) after insertion into the antibody framework.

Many peptides which could benefit from display in the context of an immunoglobulin have been identified and are known to those who practice the art, e.g., EPO and TPO mimetic peptides. Other examples include peptides that bind to receptors which are activated by ligand-induced homo-dimerization including active fragments displaying G-CSF activity, GHR activity and prolactin activity as described in Whitty and Borysenko, *Chem Biol.*, (1999) Apr 6(4):R107-18; other examples of suitable peptides include a nerve growth factor mimetic from the CD loop as described in Zaccaro et al., *Med. Chem.* (2000) 43(19); 3530-40; an IL-2 mimetic as described in Eckenberg, et al., *J. Immunol.* (2000) 165(8):4312-8; glucogon-like peptide-1 as described in Evans et al., *Drugs R.D.* (1999) 2(2): 75-94; tetrapeptide I (D-lysine-L-asparaginyl-L-prolyl-L-tyrosine) which stimulates mitogen activated B cell proliferation as described in Gagnon et al., *Vaccine* (2000) 18(18):1886-92. Peptides which exhibit receptor antagonistic activity are also contemplated. For example, N-terminal peptide of vMIP-II as an antagonist of CXCR4 for HIV therapy as described in Luo et al.,

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Biochemistry (2000) 39(44):13545-50; antagonist peptide ligand (AFLARAA) of the thrombin receptor for antithrombotic therapy as described in Pakala et al., *Thromb. Res.* (2000) 100(1): 89-96; peptide CGRP receptor antagonist CGRP (8-37) for attenuating tolerance to narcotics as described in Powell et al., *Br. J. Pharmacol.*

5 (2000) 131(5): 875-84; parathyroid hormone (PTH)-1 receptor antagonist known as tuberoinfundibular peptide (7-39) as described in Hoare et al., *J. Pharmacol. Exp. Ther.* (2000) 295(2):761-70; opioid growth factor as described in Zagon et al., *Int. J. Oncol.* (2000) 17(5): 1053-61; high affinity type I interleukin 1 receptor antagonists as disclosed in Yanofsky, et al., *Proc. Natl. Acad. Sci. USA*, Vol. 93, pp. 7381-7386, July

10 1996 and Vigers, et al., *J. Biol. Chem.*, Vol 275, No 47, pages 36927-36933, 2000; and acid fibroblast growth factor binding peptide as described in Fan et al., *IUBMB Life* (2000) 49 (6) 545-48. Peptides can also be discovered using methods familiar to those skilled in the art. In order to identify a region of a protein that is involved in a specific biological function, a survey of the shorter peptide fragments making up that

15 protein may reveal the linear peptide epitope responsible. Alternatively by surveying libraries of random peptides, a peptide that represents an optimal linear epitope or a discontinuous epitope may be discovered that mimics the activity of the natural protein. One method for selection is termed peptide phage-display. In this approach, a random peptide epitope library is generated so that peptides are present on the .

20 surface of a bacteriophage particle. These collections, or libraries, of peptides can then be surveyed for those able to bind to a specific immobilized target protein. (Pasqualini, R. et al., *j. Cell Biol.*, 130, 1995, 1189-1196; Wrighton, N.C., et al., *Science*, 273, 1996, pages 458-463; Cwirla, S.E., et al., *Science*, 276, 1997, pages 1696-1699; Koivunen et al, *J. Biol, Chem.*, 268, 1993, pages 20205-20210; Koivunen

25 et al., *Bio/Technol.*, 13, 1995, pages 265-270; Healy et al., *Biochem.*, 34, 1995, pages 3948-3955; Pasqualini et al., *J. Cell Biol.*, 130, 1995, pages 1189-1196). Alternative peptide selection systems are also possible including cell surface display and ribosomal display.

Peptide mimetics used in accordance with this description are generally less

30 than or equal to the number of amino acid residues that make up a CDR region, although they could be longer.

Any antibody can serve as a scaffold sequence, however typically human antibodies are chosen as human therapeutics is one of the ultimate objectives. Human or humanized antibodies are less likely to cause an adverse immune response in a human patient. The major criteria in selecting an antibody to serve as a

5   framework for insertion of a peptide, is that the replacement of one or more CDRs of the antibody with the peptide must change the antigen specificity. The antibody can be a complete antibody or an Fab, scFv or F(ab')$_2$ fragment or portion thereof.

Alternatively, a library of antibodies can have one or more heavy and/or light chain CDRs replaced with a desired peptide. The resulting library can then be

10   screened to identify antibodies having a desired activity. It should be understood that randomization with in the substituted peptide can also be provided to generate an antibody library.

A useful antibody is the anti-tetanus toxoid (TT) Fab, as it is human and because modification of the HCDR3 is sufficient to change the antigen specificity of

15   the antibody (Barbas et al., *J. Am. Chem. Soc.*, 116, 1994, pages 2161-2162 and Barbas et al., *Proc. Natl. Acad. Sci. USA*, 92, 1995, pages 2529-2533).

Grafting of the DNA sequence of the peptide of choice into an antibody so as to replace the CDR(s) of an antibody with the peptide sequence is carried out using recombinant DNA techniques known to those skilled in the art.

20   Examples of methods which can be utilized to graft a desired peptide having biological activity in place of a CDR region include, but are not limited to, PCR overlap, restriction enzyme site cloning, site specific mutagenesis and completely synthetic means. For a description of techniques involving overlap PCR, see, e.g., Example 1 herein. Site specific mutagenesis can be accomplished in several ways. One is

25   based on dut/ung Kunkel mutagenesis (Kunkel, T.A., *Proc. Natl. Acad. Sci.* (1985) vol. 82, pp. 488-92). The Muta-Gene *in Vitro* Mutagenesis kit is available from BioRad based on this methodology (cat. # 170-3581 or 170-3580). Several PCR amplification based mutagenesis approaches are also commercially available such as Stratagene's QuickChange Site-Directed Mutagenesis Kit and the ExSite PCR-based Site-Directed

30   Mutagenesis Kit. Another non-PCR method is available from Promega as the GeneEditor *in vitro* Site-Directed Mutagenesis System. Completely synthetic means

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are also well-known and described, e.g., in Deng, et al., *Methods Mol. Biol.* (1995) 51:329-42; Kutemeler et al., *Biotechniques*, (1994) 17(2): 242-246; Shi et al., *PCR Methods Appl.*, (1993) 3(1): 46-53 and Knuppik et al., *J. Mol. Biol.*, (2000) 11:296(1): 571-86 each incorporated herein by reference. In addition, the above methods used

5   for replacing all or a portion of at least one CDR sequence can be utilized to graft a desired peptide into or adjacent to at least one native CDR sequence without replacing the original CDR sequence. In this manner, a CDR/biologically active peptide mimetic fusion construct is formed.

It is contemplated that flanking sequences may be added to the carboxyl and/or

10   amino terminal ends of the biologically active peptide. Flanking sequences can be useful to reduce structural constraints on the grafted peptide to allow it to more easily adopt a conformation necessary for biological activity. In a preferred embodiment, a flanking region including a proline is covalently attached to the carboxy terminus of the biologically active peptide to create a proline extended biologically active peptide.

15   In one embodiment, a flanking region can be generated by randomizing two amino acid positions on each side of the peptide graft in order to determine the best sequence. In this manner, a library having members with multiple varied sequences can be generated. The resulting constructs are then tested for biological activity as described below by, e.g., panning techniques. Recombinant proteins can be

20   generated that have random amino acids at specific positions. This can be accomplished by modifying the encoding DNA. When introducing randomization at a specific amino acid's codon position, a preferable deoxyribonucleotide "doping strategy" is $(NNK)_x$ in order to cover all 20 amino acids and to minimize the number of encoded stop codons. Accordingly, N may be A, C, G, or T (nominally equimolar), K is

25   G or T (nominally equimolar), and x is typically up to about 5, 6, 7, or 8 or more, thereby producing libraries of mono-, di-, tri-, quadra-, penta-, hexa-, hepta-, and octa-peptides or more. The third position may also be G or C, designated "S". Thus, NNK or NNS (i) code for all the amino acids, (ii) code for only one stop codon, and (iii) reduce the range of codon bias from 6:1 to 3:1. There are 32 possible codons

30   resulting from the NNK motif: 1 for each of 12 amino acids, 2 for each of 5 amino

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acids, 3 for each of 3 amino acids, and only one of the three stop codons. Other alternatives include, but are not limited to:

$(NNN)_x$ which would provide all possible amino acids and all stops;

$(NNY)_x$ eliminates all stops and still cover 14 of 20 amino acids;

5      $(NNR)_x$ covers 14 of 20 amino acids; and

$(NNS)_x$ covers all 20 amino acids and only one stop.

The third nucleotide position in the codon can be custom engineered using any of the known degenerate mixtures. However, the group NNK, NNN, NNY, NNR, NNS cover the most commonly used doping strategies and the ones used herein.

10   The collection of engineered antibodies that are created during this process can be surveyed for those that exhibit properties of the peptide as, e.g., phage displayed antibodies, essentially as has been described in Barbas, C.F., III, Kang, A.S., Lerner R.A., and Benkovic, S.J., Assembly of combinatorial antibody libraries on phage surfaces: the gene III site, *Proc. Natl. Acad. Sci. USA*, 88, 1991, pages 7978-7982

15 incorporated herein by reference. This technology allows recombinant antibodies (as complete antibodies, Fab F(ab')$_2$, or scFv) to be expressed on the surface of a filamentous bacteriophage. That same phage will have within it the genes encoding that specific antibody.

It is contemplated that any other known method of introducing randomization

20 into a sequence may be utilized herein. For example, error prone PCR can introduce random mutations into nucleic acid sequences (See, e.g., Hawkins et al., J. Mol. Biol, (1992) 226(3): 889-96). Briefly, PCR is run under conditions which compromise the fidelity of replication, thus introducing random mutations in sequences as those skilled in the art would accomplish. After generation of such random mutants, they can be

25 placed into phage display formats, panned and thus evaluated for activity. Likewise, particular bacteria known to provide random mutations of genes, such as Epicurian Coli® XL1-Red Competent cells (commercially available from Stratagen, La Jolla, CA.), which do so during plasmid replication can be utilized to provide random mutants which are then screened for biological activity in accordance with the present

30 disclosure.

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It is also contemplated that randomization may be introduced at any point in the nucleotide sequence after incorporation of an active peptide into the antibody or fragment thereof to alter the overall biological activity of the antibody. In this manner, not only can alterations be made in the biological activity of a peptide mimetic by

5   causing mutations within the peptide's sequence, but mutations in the surrounding scaffold can be incorporated with the resulting constructs being assayed for alterations in biological activity or expression. Indeed, it is contemplated that libraries having repertoires of multiple constructs resulting from such randomization can be generated and assayed.

10   Single chain libraries can be utilized in accordance with the present disclosure because an entire binding domain is contained on one polypeptide. The light chain variable region is separated from heavy chain variable region by a linker region. The use of short linkers (< 11 amino acids) favors a dimeric complex where V_H of one ScFv associates with V_L of another ScFv molecule and visa versa, these molecules are

15   termed diabodies (Kortt, A.A., Malky, R.L., Caldwell, J.B., Gruen, L.C., Ivanci, N., Lawrence, M.G. et al. *Eur. J. Biochem.* 221:151-157, 1994). This is because folding of monomeric ScFv is impaired with linkers < 11 amino acids (Alfthan, K., Takkinen, K., Sizman, D., Soderlund, H., and Teeri, T.T. *Protein-Eng.* 8:725-731, 1995). Longer linkers (> 11 amino acids) favors folding of monomeric ScFv into a single antigen

20   binding domain, thus precluding dimer formation.

One useful phage display vector is pRL4 which is also known as pComb 3X (see Fig. 1). This vector enables display of chimeric expression products on the surface of packaged phagemid particles. pRL4 is a modified version of pComb3H (Barbas, C.F. III and Burton, D.R. 1994. Monoclonal Antibodies from Combinatorial

25   Libraries. Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor, N.Y.; Burton, D.R.; Barbas, C.F. III. *Advances in Immunology* 57:191-280, 1994; Lang, I.M., Chuang, T.L., Barbas, C.F. 3rd, Schleef, R.R. *J. Biol. Chem.* 271: 30126-30135, 1996; Rader and Barbas, Phage Display, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000). The design of pRL4 allows for

30   dimerization of scFv antigen binding domains on the phage surface and in soluble form as detailed below. When the plasmid is transformed into a *supE* bacterial host

such as ER2537 (F' *Sup E*, New England Biolabs, Beverly, MA), the amber mutation is suppressed approximately fifty percent of the time. In this way half of the expressed scFvs are fused with the filamentous phage gene III protein (amino acids 230-406) and the other half will be terminated just prior to gene III to produce soluble scFv. Both the

5   scFv-pIII fusion and soluble scFv products have the *Omp A* signal sequence and will be transported to the periplasm where they will be able to form dimeric scFv complexes, termed diabodies (Kortt, A.A., Malby, R.L., Caldwell, J.B., Gruen, L.C., Ivanci, N., Lawrence, M.C. et al. *Eur. J. Biochem.* 221: 151-157, 1994). Diabodies are expected to fold such that the V_H of one scFv will pair with the V_L of a second scFv-pIII

10   resulting in divalent antibody fragments. In a non-*sup E* host, such as TOP1OF' (InVitrogen, Carlsbad, CA), the amber stop codon is recognized yielding soluble scFv diabodies.

In the final single chain expression construct in pRL4, the single chain antibody fragments are cloned downstream of the *E. coli* lacZ promoter, ribosome binding site,

15   and *omp* A leader sequence. These elements allow induction of expression by IPTG, and the secretion out of the cell via the *omp* A leader sequence when expressed in the suppressor strain ER2537. The single chain fragments are fused in frame with filamentous phage gene III (gIII) sequences (amino acids 230-406). The gIII protein product, pIII, is a minor coat protein necessary for infectivity. Upon promoter induction

20   by IPTG, the single chain antibody-pIII fusion is synthesized and transported to the bacterial periplasmic space. In the periplasmic space, the scFv-gene III fusion proteins are inserted into the membrane. Upon superinfection with helper phage, these fragments are exported out of the cell on the surface of phage as pIII-antibody fragments. Other possible proteins to be used for fusion on the surface of phagemids

25   include filamentous coat protein pVIII and other coat proteins.

Fab fragment libraries, that maintain the native antigen recognition site, are useful to ensure that affinity is maintained.

In the final hybrid Fab expression construct in pRL4, the light and heavy chains are cloned as a single Sfil fragment. In this way, the light chain fragments are cloned

30   downstream of the *E. coli* lacZ promoter, ribosome binding site, and *omp* A leader sequence. These elements allow induction of expression by IPTG, and secretion out

of the cell via the *omp* A leader sequence. The light chain fragments are followed by a stop codon, a second ribosome binding site, the *E. coli pel* B leader sequence and heavy chain. Hybrid heavy chain genes are fused in frame with filamentous phage gene III (gIII) sequences (amino acids 230-406). An amber stop codon is present at

5   the fusion junction. In a *sup E* bacterial host such as ER2357 (New England Biolabs, Beverly, MA), the amber mutation is suppressed. Upon promoter induction, a single polycistronic message is transcribed and translated as two polypeptides, a light chain and a heavy chain-gene III fusion protein. Following synthesis the polypeptides are transported to the bacterial periplasmic space as directed by the leader sequences. In

10   the periplasmic space the heavy chain-pIII fusion proteins are inserted into the membrane, and the light and heavy chains are associated covalently through disulfide bonds, forming the antigen binding sites. The human constant region CH1 and C_L sequences include the cysteines that form the disulfide bond between heavy and light chains. Upon superinfection with helper phage, these fragments are exported out of

15   the cell on the surface of phage as Fab-cpIII fusion. In a non-*sup E* host, such as TOP1OF' (Invitrogen, Carlsbad, CA), the amber stop codon is recognized yielding soluble Fab fragments. Important features of the pRL4 phage display system used include a purification His 6 tag, an HA epitope tag following the heavy chain, as well as a suppressible amber stop codon which is located between the heavy chain and the

20   phage gene III. The HA tag is recognized by HA.11 antibody (Babco, Berkeley, CA) and 12CA5 antibody (Roche Molecular Biochemical, Indianapolis, Ind.). The His6 tag allows affinity purification of antibody fragments by Nickel-chelate chromatograph (Qiagen, Valencia, CA). The amber stop allows for quick conversion from a fusion Fab-cpIII product (for incorporation on the phage coat) when the stop is suppressed, to

25   the soluble Fab which is made in a non-suppressor bacterial host.

Selection involves isolating from the library the best candidates that specifically bind to the peptides target molecule and display biological activity.

The phage expressing antibody fragments on their surface can be produced and concentrated so that all members of a library can be allowed to bind to the target

30 .   molecule. The target molecule can be immobilized on a microtiter dish, on whole cells, the membranes of whole cells, or present in solution. Non-specific Ab-phage are

20

washed away, and bound phage particles are released from the antigen, often by the use of low pH. The recovered Ab-phage are infectious and so can be amplified in a bacterial host. Typically, multiple rounds of this sort of selection are performed. Individual antibody fragment clones can then be analyzed as soluble Fabs or scFvs for

5   identification of those that specifically recognize the target molecule.

Prior to any selection strategy, initial libraries are electroporated into host cells, such as ER2537. Library cultures are grown to log phase and superinfected with helper phage, such as VCSM13, a commercially available helper phage (Stratagene, La Jolla, CA). Superinfection provides the remaining phage components needed for

10   packaging plasmids into phagemid particles. Alternatively, phage display without the use of helper phage may be utilized. Following overnight growth, phagemids in the culture supernate are precipitated with polyethylene glycol (PEG). PEG precipitated phage are used in panning (solid phase cell surface, internalization and membrane), FACS sorting, or magnetic sorting to purify specific binding antibodies from non

15   specific binders.

In cell based panning, antibody-phage libraries are incubated with target cells, and the non-adherent phage are removed with multiple washes. A typical panning protocol is as follows:

1. Block phage particles with PBS + 1%BSA or 10% FBS + 4% milk powder +

20   NaN_3, (except when internalized antibodies are assayed).

2. Add target cells to blocked phages (approximately 5 X 10^6 cells).

3. Mix and rotate slowly at 4° C or 37 ° C.

4. Wash cells twice with 1 ml ice cold PBS/1%BSA/NaN_3 or room temperature

25   PBS/1%BSA/NaN_3.

5. Specific antibody-phage bound to cells can be eluted by low pH, for example with 76 mM citric acid ph 2.5 in PBS for 5 to 10 minutes at room temperature.

6. Neutralize eluted phage with 1M Tris-HCl pH 7.4.

7. After neutralization, antibody-phage can be used to infect ER2537 bacteria

30.   and amplify during overnight growth for the next round of panning.

Generally, 3-4 rounds of panning are performed on each library. Phage ELISAs using commercially available secondary antibody (sheep anti-M13 antibody-HRP) or soluble antibody ELISAs using a commercially available HA. 11 antibody (Babco, Berkeley, CA) that recognizes the HA tag incorporated into each antibody from PRL4

5   sequences, can be performed following each round of panning to allow estimation of the enrichment of binding antibodies over non-binders. Following the last round of panning, the antibody-phage can be picked as single colonies from agar plates, grown as monoclonal antibody-phage and screened by ELISA for identification of specific binders. FACS analysis may also be utilized. Specifically the antibody-phage are

10   infected into Top10F' bacteria and plated for single colonies. Single colonies are picked form agar plates, grown and induced with IPTG. Soluble antibody is screened by ELISA for identification of specific binders. Screening can be done against live cells, against intact, mildly fixed target cells, or recombinant protein(s).

     Methods for whole cell panning have been described previously (Siegel, D.L.,

15   Chang, T.Y., Russell, S.L., and Bunya, V.Y. 1997. *J. Immunol. Methods* 206:73-85 incorporated herein by reference). Other techniques for selection which can be applied include fluorescent activated cell sorting (FACs). Alternative methods for selection using libraries include, but are not limited to, ribosome display and plaque hybridization to a labeled antigen.

20   Following panning to isolate high affinity antibody binders, bioassays for functional screens of agonist antibodies can be carried out. Dimerization is often a prerequisite for activation of many receptors and thus bioassays focus on agonist antibodies that stimulate receptors via promotion of dimerization. As previously described, single chain multivalency is approached in linker design. Fab fragment

25   multivalency can be approached in a number of ways. A number of recent reports in the literature have shown success in dimeric antibody fragment formation which is applicable to phage display (DeKruif, J., and Logtenberg, T. 1996. *J. Biol. Chem.* 271:7630-7634, Pack, P., and Pluckthun, A. 1992. *Biochemistry* 31:1579-1584, and Holliger, P., and Winter, G. 1993. *Current Opin. Biotech.* 4:446-449). Divalent Fabs

30   can be created in at least two ways. In one approach dimerization is achieved by addition of a dimerization domain to pRL4, forming pRL8 (See Figs. 6A-C, 7 and 8).

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There are a number of dimerization domains (lexA, Zn fingers, fos, jun etc.) that can be utilized in these vectors to obtain multivalency of Fab fragments. Dimerization domains are selected from, but not limited to, the following: jun (DeKruif, J. and Logtenberg, T. *J. Biol. Chem.* 271:7630-7634, 1996; Kostelny, S.A., Cole, M.S., and Tso, J.Y. *J.Immunol.* 148:1547-1553, 1992) the LexA dimerization region (Kim, B. and Little, J.W. *Science* 255:203-206, 1992), the yeast GCN4 dimerization domain (van Heeckeren, W.J., Sellers, J.W., Struhl, K. *Nucleic Acids Res.* 20:3721-3724,1992), Gin invertase from the bacteriophage Mu (Spaeny-Dekking, L., Schlicher, E., Franken, K., van de Putte, P., Goosen, N. *J. Bacteriol.* 34:1779-1786, 1995), *E. coli* NTRC protein dimerization domain (Klose, K.E., North, A.K., Stedman, K.M., Kustu, S. *J. Mol. Biol.* 241:233-245, 1994), and HSV-1 ICP4 dimerization domain (Gallinari, P., Wiebauer, K., Nardi, M.C., Jiricny, J. *J. Virol.* 68:3809-3820, 1994) all incorporated by reference. Also, a high temperature dimer domain from thermus organisms can be utilized (MacBeath, G., Kast, P., Hilvert, D., *Biochemistry* 37:100062-73, 1998 and MacBeath, G., Kast, P., Hilvert, D., *Science* 279:1958-61, 1998). These are functional domains that when incorporated into a molecule allow for dimerization to occur. In addition, dimerization can be achieved in cells through the use of full IgG vectors, or dimerization domains such as CH3 dimerization domains. Those of ordinary skill in the art are familiar with these and other dimerization domains and their use to dimerize proteins.

Additional methods that may be utilized to generate antibody constructs which contain at least two binding sites are known. The antibody or antibody fragments created by each of these approaches could be utilized for testing agonistic antibody activity as described in Example 1 below for whole IgG produced in mammalian cells. These methods include chemical dimerization of Fab, pegylation of Fab, production of Fab'2, generation of whole IgG in bacterial cells, and use of diabodies (scFvs). Importantly, any of the antibody forms generated for analysis of agonistic activity could be used as the final therapeutic product.

Chemical dimerization may be also achieved using a variety of chemical crosslinking reagents. For example, SMCC (Succinimidyl trans-4 (maleimidylmethyl) cyclohexane-1-carboxylate) from Molecular Probes (Eugene, Oregon), Cat # S-1534.

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This reagent will modify primary amino groups in the antibody. After incubating the antibody with the SMCC at room temperature, the reaction is run over a PD-10 column. This maleimide derivitized Fab can be added to either a second Fab or a separate batch of the same Fab that has been treated with TCEP [(Tris (2-

5   carboxyethyl) phosphine, hydrochloride): Molecular Probes Cat #T-2556] to reduce the thiol groups to SH. The reduction reaction is carried out in the dark for 15 minutes. The conjugation of the maleimide Fab and the thiol reduced Fab occurs at a 1:1 ratio. Dimers are isolated by passing the reaction over a sephadex 200 gel filtration column. Other chemical linkers known to those skilled in the art may be used for dimerization.

10   Production of an Fab' that has an extra cysteine residue engineered into the hinge region has been described, e.g., in U.S. Patent 5,677,425 and Carter, et al.,*BioTechnology*, Vol 10, Feb. 1992, pages 163-167 the disclosures of which are hereby incorporated by reference. That thiol site can be used for conjugation to moieties such as polyethyleneglycol (PEG). Pegylation technology is known, for

15   example, see Koumenis et al, Int. J. Pharm. (2000) 198(1): 83-95, incorporated herein by reference, which makes it possible to link two Fab' molecules together using PEG coupling.

Technology for bacterially producing Fab'2 involves cloning the human IgG hinge region, and optionally part of the CH2, as part of the Fd which includes

20   additional cysteines and is described, e.g., in Better, et al., PNAS USA (1993) 90(2): 457-61, incorporated herein by reference. The additional thiol groups on the Fd hinge can interact and cause two Fab' molecules to dimerize, creating a Fab'2. Fab'2 can be purified directly from the bacterial cells. Additionally, undimerized Fab' from the bacteria can be isolated and chemically converted to Fab'2.

25   As described earlier, the variable regions of the antibody can be cloned as a single chain wherein the variable light (VL) is connected to the variable heavy (VH) by a linker region. If that linker region is short (for example 5-7 amino acids), the folding of the scFv will favor association of two scFvs where the VL of one scFv paris with the VH of the second scFv. In this manner, two antigen binding sites are

30   presented on the diabody.

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Antibody constructs which contain two binding sites may be generated using any of these methods in order to test agonist activity and/or be used as the final therapeutic product.

Following the panning or sorting steps of Fab libraries, the library of panned molecules are restricted with Sac I and *Spe* I and cloned into pRL8. Subcloning to pRL8 vector individually or *en masse* following FACS sorting or panning allows expression of dimeric soluble binding Fabs for analysis in bioassays. In pRL8, the antibody fragments are transported to the periplasmic space and form dimers there. The advantage of this approach is that it permits panning of monomeric Fab fragments, favoring high affinity Fabs.

Another approach uses a secondary antibody. pRL4 has the hemagglutinin decapeptide tag recognized by the commercially available HA. 11 antibody (Babco, Berkeley, CA). Fabs identified in FACS sorting or panning to be tested in bioassay are preincubated with HA. 11 which will promote dimerization, prior to addition to bioassays.

Once binding scFv's or Fabs are identified by panning or another selection method, the individual clones, each expressing a unique dimerized antibody fragment on the phage surface are tested for proliferation, differentiation, activation or survival effects on target cells. In addition, soluble dimerized antibody are examined in bioassays.

Biological Assays for Screening for TPO-like Activity

1. Colony formation assays - Megakaryocytic colonies from bone marrow (Megacult C Kit from Stem Cell Technologies Inc., Vancouver BC, Canada).

2. Proliferation assays - proliferation of Ba/F3 cells (Cwirla et al. 1997, Science, Vol. 276 pages 1696-1699). The Ba/F3-mpl cell line was established (F. de Sauvage *et al.*, Nature, 369:533 (1994)) by introduction of the cDNA encoding the entire cMpl receptor into the IL-3 dependent murine lymphoblastoid cell line Ba/F3. Stimulation of proliferation of Ba/F3-mpl cells in response to various concentrations of antibodies or TPO was measured by the amount of incorporation of ^{3}H-thymidine as previously described (F. de Sauvage *et al.*, *supra*).

3. Phosphorylation assays - phosphorylation of JAK2 (Drachman et al., J. Biol. Chem., (1999), Vol. 274, pages 13480-13484).

4. Transcriptional based assays - Transiently co-transfect full length cMpl receptor with c-Fos promoter luciferase reporter construct. 24 hour post transfection starve the cells in 0.5% FCS for 24 hours. Stimulate the cells, harvest after 6 hours and take luciferase readings (see also Example 1, Biological Assays section).

Biological Assays for Screening for EPO-like Activity

1. Bone marrow erthroid colony formation in Methylcellulose (Wrighton et al., Science, 1996, Vol. 273 pages 458-463).

2. TF-1 cell (Human erythroleukemia cell line) proliferation. TF-1 cells express both full length and a truncated form of the Epo-R. (J.Cell Physiol., 1989, Vol 140, pages 323-334).

3. The EPO receptor couples directly to JAK2 kinase to induce tyrosine phosphorylation. Epo induces cFos in TF-1 cells. c-Fos transcriptional activation. (Witthuhn et al., Cell, (1993), Vol. 74, pages 227-236).

A number of bioassays can be used in high-throughput screening. Those of ordinary skill in the art are familiar with these and other suitable bioassays. Several non-radioactive assays have been developed in which either DNA synthesis or enzyme activity can be analyzed. For example, an MTT cell proliferation assay (Promega Corporation, Madison, WI) that is based on an assay described by Mosmann (Mossman, T. 1983. *J. Immunol. Methods* 65:55-57 incorporated herein by reference) can be used. This protocol is fast and easy. In the assay, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), a tetrazolium salt, is converted into a blue formazan product by mitochondrial dehydrogenase activity in living cells. The dehydrogenase content, and therefore the amount of colored product produced, is proportional to cell number. The colored product is detectable in an ELISA plate reader at 570nm. Assays are performed in triplicate, *en masse* in 96 well microtiter plates. Briefly, target cells are plated in 100 μl aliquots in culture medium in 96-well plates. Following addition of various concentrations of antibodies, cells are incubated for 48-

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72 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. MTT is added to each well, and proliferation monitored via ELISA plate reader.

For example, in proliferation assays using TF-1 cells, bacterial cells containing phagemids expressing antibodies are grown overnight at 37°C in 96 well deep well plates in 1 ml of a media that is a mixture of mammalian cell media and bacterial media (in the case of TF-1 cells: RPMI 2.7/SB 0.3/Carb 100ug/ml). TF-1 cells are a human bone marrow erythroleukemia cell line that responds to multiple cytokines (Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.F., Miyazono, K., Urabe, A., Takaku, F., *Cell Physiol.* 140:323-334, 1989; Kitamura, T., Tojo, A., Kuwaki, T., Chiba, S., Miyazono, K., Urabe, A., Takaku, F., *Blood* 73:375-380, 1989; Kitamura, T., Takaku, F., Miyajima, A., *Int. Immunol.* 3:571-577, 1991) On the following day, the overnight cultures are subcultured 1/10 to fresh trays, and placed at 37°C for 2 hours. Following induction with IPTG at 37°C for 4 hours, the plates are centrifuged at 2000 rpm/15' at room temperature. 50 ul each culture supernate are filtered in 96 well filter trays (Millipore) to sterile 96 well assay plates. Mammalian cells are prewashed to remove growth factor and resuspended at a concentration of 1×10^5 cells/ml. 50 ul cells are added to each well. Assay plates are incubated in 37°C/5% CO_2 incubator for 72 hours. At 72 hours, the trays are developed by adding 40 ul media/MTS/PMS per well. MTS is an improved more soluble version of MTT. Both assays are based on the cellular conversion of tetrazolium salt. A MTS proliferation assay kit (catalogue number G5421) can be purchased from Promega, Inc. (Madison, WI). Plates are kept at 37°/CO_2 incubator and read at OD_{490} at 1 hour, 4 hours, 8 hours with microplate reader.

The activities of cytokines are often synergistic. Synergy could be manifested through the binding of ligands to two different receptors which then sends the correct signal, or via a priming effect whereby interaction of ligand/receptor primes the cell to respond to a second cytokine. Furthermore, cytokines that act early in lineage development are more often synergistic than cytokines that act at later stages in a developmental pathway. Therefore, suboptimal concentrations of growth factors can be used in these bioassays to examine synergism. Conditions for suboptimal concentrations are determined for each assay. This is done by adding serial dilutions

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of growth factors, individually and as a mixture, to the assays and determining the levels below which a single factor does not promote a response compared to the mixture, and the level below which the mixture does not promote a response in the bioassay. Bone marrow stromal cells can also be added in bioassays to provide other

5   necessary factors that may play a role in a synergistic response.

In addition, cell proliferation can be examined by monitoring DNA synthesis. A non-radioactive, colorimetric assay that examines 5-bromo-2'-deoxy-uridine (BrdU) incorporation (Roche Molecular Biochemicals, Indianapolis, IN) can be performed in microtiter plate format. Here, cells are cultured in 96-well plates and incubated with

10 BrdU and sub-optimal concentrations of cytokines. The amount of BrdU is determined after labeling with a peroxidase labeled anti-BrdU antibody. Final results are analyzed by ELISA plate reader at 405nm.

A radioactive mitogenesis assay that measures the rate of DNA synthesis as an indication of proliferation (Raines and Ross, *Methods of Enzymol.* 109: 749-773,

15 1985) can also be used. In these assays, changes in rate of incorporation of [^{3}H]-thymidine in target cells is examined. Again, these assays permit concurrent and rapid screening of many antibody fragments. They have been widely used as a convenient method of assessing the stimulatory and inhibitory effects on the growth of many different cells. Cells are cultured in suspension until they reach exponential growth

20 rate. Cells are then washed free of the medium in which they were cultured, and replated in fresh medium. Cells are aliquoted into 96 well plates in a total volume of 100 ul at a concentration of about 1-2 x 10^5 cells/ml. Dilutions of phage supernatant, soluble dimerized Fab or ScFv antibodies are added and cells are incubated for 18-48 hours in a gassed CO_2 incubator at a temp of 37°C. Following incubation,

25 [^{3}H]thymidine (937kBq) is added to each well and incubated for a further 4 hours. The cells are then removed from the incubator and counted directly in a bench top microplate scintillation counter such as Packard Top Count NXT Instrument (Packard, Meriden, CT). Alternatively cells can be serially transferred to GF/C filters on a Millipore cell harvester (Millipore, Bedford, MA) or similar apparatus. Radioactivity

30 associated with acid-insoluble material retained on the filter is then determined. Dilutions of commercially available growth factors are applied to positive control wells.

Negative controls would include supernatants from cells carrying non-insert containing plasmids or irrelevant antibodies treated similarly. The relative growth promoting activities of the standard and the diluents of the phage supernatants under test are compared to quantify the growth promoting activity in the sample.

5   Activation can be tested for by assaying second messengers or by transcriptional readout assays.

Survival can be assayed, for example, by monitoring apoptosis using assays such as tunnel assays or by other methods known to those who practice the art.

Other useful assays to analyze cellular signal transduction, the activity of
10   kinases and phosphatases and ultimately cellular activities as a result of agonist activity include measurement of the generation of second messengers, e.g. cAMP, Ca+ +, diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP3). Measurement of spikes in intracellular calcium concentration, intracellular pH and membrane potential in high throughput screening assays can be performed using instruments such as the
15   FLIPR Fluormetric Imaging Plate Reader System (Molecular Devices, Sunnyvale, CA). A number of fluorescent probes are available for examination of second messenger concentrations (Molecular Probes, Eugene OR). Measurement of concentrations of second messengers can also be done on the single cell level (DeBernardi, M.A. and Brooker, G. *Proc. Natl. Acad. Sci USA* 93:4577-4582, 1996). In addition, assays that
20   examine other signaling events such as phosphorylation, apoptosis or levels of RNA or protein of specific genes would be useful. For example. most cytokines have been shown to activate the enzyme PI 3-K (reviewed in Silvennoinen, O., Ihle, J.N. Signaling by the Hematopoietic Cytokine Receptors, R.G. Landes company, Austin, TX 1996). Furthermore, the Jak family of tyrosine kinases have been shown to be central
25   mediators for cytokine receptor signaling (Ihle, J.N., Witthuhn, B.A., Quelle, *F. W. Annu. Rev. Immunol.* 13:369-398, 1995). In addition, several other tyrosine kinases, e.g., members of the Src family, are activated in response to certain cytokine stimulations. In the case of RNA or proteins, e.g., c-Jun and c-Fos are rapidly and transiently upregulated upon cytokine stimulation, while c-Myc induction is slower.
30   These proteins are required for G1 transition and proliferation (reviewed in Silvennoinen, O., Ihle, J.N. Signaling by Hematopoietic Cytokine Receptors, R.G.

Landes Company, Austin, TX 1996). High throughput screens that detect increases in these transcripts could be utilized.

In transcriptional read out assays, changes in the transcription of specific genes are observed following exposure of cells to a growth factor·' or growth factor mimetic

5   (agonist or inhibitory antibody). For example, in a myc read-out assay, cells such as IL-3 dependent FDCP-mix cell line is starved of IL-3 growth factor for 8 hours, followed by exposure to growth factor mimetics, or native growth factors for 2 hours at 37°C. At this time, the cells are harvested, RNA is isolated, and reverse transcriptase-polymerase chain reactions (RT-PCR) are performed with primers specific for the myc

10 gene. The RT-PCR reactions are electrophoresed in horizontal agarose gels for quantitation of PCR product. In this case expression of a single gene is being monitored.

Alternatively assay for changes in expression of genes can be monitored using CHIP technology, agonist antibodies could be identified under·conditions of high probe

15 sensitivity and a dynamic range. In this way, up to 10,000 or more could be analyzed for changes in expression. Desired genes that could be monitored could include c-myc, c-jun, NF-κB, among others. These genes are downstream of various signal transduction pathways and their expression should change upon a mitogenic response. In one type of commercially available CHIP (Affymetrix, Santa Clara, CA),

20 oligonucleotides from desired test genes can be printed out onto glass surface. Target cells are exposed to test agonist antibodies. RNA is isolated from the cells exposed to test agonist antibodies, copied to cDNA, and *in vitro* transcribed in the presence of biotin. Hybridization of *in vitro* transcribed, biotinylated mRNA is used as probe in the arrays. Chips are then scanned to determine genes that show increases in

25 transcription upon exposure to test agonist antibodies. In another version of CHIP technology (Incyte, Palo Alto, CA), the amount of DNA is not normalized on the glass, therefore, one would set up a competitive hybridization. RNA is isolated from the cells before and after exposure to agonist. cDNA is made from each sample whereby one cDNA reaction has one label incorporated, for example, Cy-3, and the other cDNA

30 population has a different label incorporated, for example Cy-5. Signals are detected and compared on a dual laser scan to collect images.

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Visual assays can also be used such as traditional methylcellulose colony forming assays (Stem Cell Technologies, Vancouver BC, Canada). In these assays, colony growth, and morphological changes are scored via light microscope. Visual examination for proliferation or differentiation effects in semi-solid agar cultures or methylcellulose can be performed using the appropriate cell line. Williams Hematology 5 (eds. E. Beutler, M.A. Lichtman, B.S. Coller L T.J. Kipps), McGraw-Hill, Inc., pp L22-L26, 1995). Addition of methylcellulose allows clonal progeny of a single progenitor cell to stay together and facilitates the recognition and enumeration of distinct colonies. All necessary components are added to a basic methylcellulose medium (such as Iscove's MDM, BSA, (-mercaptoethanol, L-glutamine) except colony-stimulating factor supplements and test antibodies (phage supernatants, soluble antibodies) are added to see if they can substitute for growth factors. Cells in methylcellulose culture are incubated for 10-12 days following the addition of antibodies in a 37°C humidified atmosphere of 5% CO_2 in air. After 10-12 days of incubation, colonies are counted using an inverted microscope. After another 8-10 days, colonies are counted again. Comparisons are made between media containing antibodies and controls with and without growth factors. In addition, colonies can be picked from methylcellulose and individual cells examined cytologically by staining with Wright's stain (see Atlas of Hematological Cytology, F.G.J. Hayhoe and R.J. Flemans, Wiley-InterScience 1970).

The receptor-binding affinities of antibody fragments can be calculated (Lfas & Johnson, 1990) from association and dissociation rate constants measured using a BIACORE surface plasmon resonance system (Pharmacia Biosensor). A biosensor chip is activated for covalent coupling of gD-mpl receptor using N-ethyl-N'''-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's (Pharmacia Biosensor) instructions. gD-mpl is buffer-exchanged into 10 mM sodium acetate buffer (pH 4.5) and diluted to approximately 30 μg/mL. An aliquot (5 μL) is injected at a flow rate of 1 μL/min to achieve approximately 400 response units (RU) of coupled protein. Finally, 1M ethanolamine is injected as a blocking agent. For kinetics measurements, 1.5 serial dilutions of antibody are injected in PBS/Tween buffer (0.05% Tween-20 in phosphate buffered saline) at 25°C using a

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flow rate of 20 μL/min. Equilibrium dissociation constants, K_d s, from SPR measurements are calculated as k_{off}/k_{on}. Standard deviations, s_{on} for k_{on} and s_{off} for k_{off}, are typically obtained from measurements with > 4 protein concentrations (k_{on}) or with > 7 protein concentrations (k_{off}). Dissociation data are fit to a simple AB->A+B model to obtain koff +/- s.d. (standard deviation of measurements). Pseudo-first order rate constant (ks) are calculated for each association curve, and plotted as a function of protein concentration to obtain kon +/- s.e. (standard error of fit).

For conversion of antibody clones into full IgGs, the coding regions for both the light and heavy chains, or fragments thereof, can be separately cloned out of a bacterial vector and into mammalian vector(s). A single vector system, such as pDR1 or its derivatives, can be used to clone both light and heavy chain cassettes into the same plasmid. Alternatively, dual expression vectors where heavy and light chains are produced by separate plasmids can be used. Mammalian signal sequences need to be either already present in the final vector(s) or appended to the 5' end of the light and heavy chain DNA inserts. This can be accomplished by initial transfer of the chains into a shuttle vector(s) containing the proper mammalian leader sequences. Following restriction enzyme digestion, the light chain and heavy chain regions, or fragments thereof, are introduced into final vector(s) where the remaining constant regions for IgG1 are provided either with or without introns. In some cases where introns are used, primer design for PCR amplifying the light and heavy chain variable regions out of pRL4 may need to include exon splice donor sites in order to get proper splicing and production of the antibodies in mammalian cells.

With either vector expression system (single or dual plasmid), the production of antibody heavy and light chains can be driven by promoters that work in mammalian cells such as, but not limited to, CMV, SV40, or IgG promoters. Additionally, the vector(s) will contain a selectable marker for growth in bacteria (such as, but not limited to, ampicillin, chloramphenicol, kanamycin, or zeocin resistance). Selectable markers for mammalian cells (such as, but not limited to, DHFR, GS, gpt, Neomyocin, or hygromyocin resistance) may also be present in the IgG vector(s), or could be provided on a separate plasmid by co-transfection.

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Those of ordinary skill in the art using known techniques would be able to synthesize antibodies in other organisms such as yeast, mammalian, insect, and plants (Carlson, J.R. and Weissman, I.L., *Mol. Cell. Biol.*, 8:2647-2650, 1988; Trill, J.J., Shatzman, A.R., Ganguly, S. *Curr. Opin. Biotechnol.* 6:553-560, 1995; Hiatt, A.,

5   Cafferkey, R. Bowdish, K. *Nature* 342: 76-78, 1989).

As stated previously, antibodies made in accordance with the disclosure herein provide increased half-life (duration of action) to the activity of small peptides or peptide mimetics such as the TPO mimetic described herein. In another aspect, the serum half-life of an antibody can itself be prolonged by making derivatives that are

10   pegylated. See, e.g., Lee, et al., Bioconjug. Chem (1999) 10(6): 973-81, incorporated herein by reference. Another advantage, e.g., of the TPO mimetic antibody described herein is that normal TPO treatment may result in generation of TPO neutralizing antibodies in patients which interfere with the activity of a patient's naturally occurring TPO. The present TPO mimetic antibody substantially reduces the likelihood that a

15   detrimental immune response will be produced toward native TPO because it has a different amino acid sequence.

The molecules encompassed by the claims can be used in diagnostics where the antibodies or fragments thereof are conjugated to detectable markers or used as primary antibodies with secondary antibodies that are conjugated to detectable

20   markers. Detectable markers, include radioactive and non-radioactive labels and are well-known to those with skill in the art. Common non-radioactive labels include detectable enzymes such as horseradish peroxidase, alkaline phosphatase and fluorescent molecules. Fluorescent molecules absorb light at one wavelength and emit it at another, thus allowing visualization with, e.g., a fluorescent microscope.

25   Spectrophotometers, fluorescence microscopes, fluorescent plate readers and flow sorters are well-known and are often used to detect specific molecules which have been made fluorescent by coupling them covalently to a fluorescent dye. Fluorochromes such as green flurorescent protein, red shifted mutants of green fluorescent protein, amino coumarin acetic acid (AMCA), fluorescein isothiocyanate

30   (FITC), tetramethylchodamine isothiocyanate (TRITC), Texas Red, Cy3.0 and Cy5.0 are examples of useful labels.

The molecules can be used in cell isolation strategies such as fluorescence-activated cell sorting (FACS) if fluorescent markers are used, In fluorescence-activated cell sorting, cells tagged with fluorescent molecules are sorted electronically on a flow cytometer such as a Becton-Dickinson (San Jose, California) FACS IV cytometer or

5   equivalent instrument. The fluorescent molecules are antibodies that recognize specific cell surface antigens. The antibodies are conjugated to fluorescent markers such as fluorescein isothiocyanate (FITC) or Phycoerythrin (PE).

Magnetic sorting is also possible. In magnetic sorting procedures, the antibody is linked directly or indirectly to magnetic microbeads. Cells are precoated with

10   antibodies that recognize cell surface molecules, e.g., receptors involved in proliferation, differentiation, activation or survival. The antibodies are attached to magnetic beads conjugated with a secondary immunoglobulin that binds to the primary antibody displaying the peptide, such as to the HA molecular tag engineered into each antibody. The cells are then removed with a magnet. Magnetic sorting can be positive

15   selection where cells of interest are bound by the antibody and hence the magnet, or negative selection where undesired cells are isolated onto the magnet.

Alternatively, radiolabeled antibodies can be used for diagnostic purposes.

Antibodies and fragments thereof disclosed herein are useful for the amplification of a variety of clinically relevant cell types. Treatment can be *in vivo* or *ex*

20   *vivo*. For example, agonist antibodies are useful to treat patients suffering from a deficiency in a cell population caused by disease, disorder or treatment related to for example suppression of hematopoiesis where less than the normal number of cells of a given lineage or lineages are present in a patient. The following represent only some examples of the conditions that can be treated with the antibodies containing

25   biologically active peptides disclosed herein, those who practice the art would be able to identify other diseases and conditions that would benefit from such treatment. For example, HIV-infected patients, patients undergoing chemotherapy, bone marrow transplant patients, stem cell transplant patients, and patients suffering from myeloproliferative disorders show subnormal levels of specific hematopoietic lineages.

30   Thrombocytopenia can be a result of chemotherapy, bone marrow transplantation or chronic disease such as idiopathic thrombocytopenia (ITP) which all

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result in low platelet levels. The present TPO mimetic antibodies can be used to treat such patients.

Patients undergoing renal dialysis often suffer from treatment related anemia with subnormal levels of red blood cells. In aplastic anemia, bone marrow suppression can cause pancytopenia or may affect only the red blood cells, the white cells, or the platelets. The disclosed antibodies will augment the armamentarium of therapeutic agents for these and other diseases and disorders characterized by deficiencies in specific cell populations, such as hematopoietic cells.

The molecules encompassed by the claimed invention can also be used for *ex vivo* proliferation and differentiation of cells. This is useful for gene therapy purposes, for example for traditional viral vector approaches, and for autologous bone marrow transplants.

In addition, certain antibodies in accordance with the present disclosure can be radiolabeled for radioimmunotherapy or conjugated to toxins to deliver such toxins to specific cell types and result in the killing of those cells.

A biologically active *c-mpl* agonist antibody capable of stimulating proliferation, differentiation and maturation of hematopoietic cells may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia *(e.g.,* aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the disclosed antibodies as well as disorders such as disseminated intravascular coagulation (DIC), immune thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, and thrombotic thrombocytopenia.

The biologically active *c-mpl* agonist antibodies disclosed herein containing the TPO mimetic peptide may be used in the same way and for the same indications as thrombopoietin (TPO). Thrombopoietin (TPO) stimulates megakaryocytopoiesis and platelet production. These antibodies are expected to have a longer half-life than

native or pegylated TPO and thus are used in indications where a longer half-life are indicated.

An example of an assay useful for determining activity of TPO mimetics is the rebound thrombocytosis assay which involves administering to mice a single injection of goat anti-mouse platelet serum to induce acute thrombocytopenia (day 0). On days 5 and 6 mice are injected with test samples. On day 8 platelet counts are determined (^{35}S incorporation into platelets).

EPO mimetic antibodies herein stimulate hematopoiesis in a manner similar to naturally occurring EPO. Such therapy is useful in treating conditions where red blood cell production is compromised such as in chronic renal failure. The biological activity of EPO mimetic antibodies may be determined using *in vitro* or *in vivo* assays.

One *in vitro* assay measures the effect of erythropoietin mimetic antibodies on erythropoiesis in intact mouse spleen cells according to the procedure of Krystal, G., *Exp. Hematol.* 11:649-660 (1983). To screen various embodiments of the EPO mimetic antibodies for activity, for example, *in vitro* or *in vivo*, the EPO mimetic antibodies can be evaluated for the extent of erythropoiesis or receptor binding. Tests to determine biological activity are well-known to those of skill in the art. For example, the biological activity of erythropoietin can be measured as described in, e.g., U.S. Pat. No. 5,614,184 and U.S. Pat. No. 5,580,853 herein incorporated by reference.

The route of antibody administration is in accord with known methods, *e.g.,* injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, subcutaneous, intraocular, intraarterial, intrathecal, inhalation or intralesional routes, topical or by sustained release systems as noted below. The antibody is preferably administered continuously by infusion or by bolus injection. One may administer the antibodies in a local or systemic manner.

The antibodies of the invention may be prepared in a mixture with a pharmaceutically acceptable carrier. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. This therapeutic composition can be administered intravenously or through the nose or lung, preferably as a liquid or powder aerosol (lyophilized). The composition may also

be administered parenterally or subcutaneously as desired. When administered systematically, the therapeutic composition should be sterile, pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art.

5   Briefly, dosage formulations of the compounds of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and may include buffers such as TRIS HCl, phosphate, citrate, acetate and

10   other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including

15   cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium and/or nonionic surfactants such as TWEEN, PLURONICS or polyethyleneglycol.

          When used for *in vivo* administration, the antibody formulation must be sterile and can be formulated according to conventional pharmaceutical practice. This is

20   readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The antibody ordinarily will be stored in lyophilized form or in solution. Other vehicles such as naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Buffers, preservatives, antioxidants and the like can be

25   incorporated according to accepted pharmaceutical practice.

          Pharmaceutical compositions suitable for use include compositions wherein one or more rationally designed antibodies are contained in an amount effective to achieve their intended purpose. More specifically, a therapeutically effective amount means an amount of antibody effective to prevent, alleviate or ameliorate symptoms of disease

30   or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in

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light of the detailed disclosure provided herein. Therapeutically effective dosages may be determined by using *in vitro* and *in vivo* methods.

An effective amount of antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the

5 condition of the patient. In addition, the attending physician takes into consideration various factors known to modify the action of drugs including severity and type of disease, body weight, sex, diet, time and route of administration, other medications and other relevant clinical factors. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the

10 optimal therapeutic effect. Typically, the clinician will administer antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

For any antibody containing a peptide, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in

15 animal models to achieve a circulating concentration range that includes the EC_{50} as determined in cell culture *(e.g.,* the concentration of the test molecule which promotes or inhibits cellular proliferation or differentiation). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the antibody molecules described herein

20 can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Molecules which exhibit high

25 therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such molecules lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of

30 administration utilized. The exact formulation, route of administration and dosage can

be chosen by the individual physician in view of the patient's condition. (See *e.g.,* Fingl *et al.,* 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the antibody which are sufficient to promote or inhibit cellular proliferation or differentiation or minimal effective concentration (MEC). The MEC will vary for each antibody, but can be estimated from *in vitro* data using described assays. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Antibody molecules should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake, the effective local concentration of the antibody may not be related to plasma concentration.

A typical daily dosage might range from about 1μ/kg to up to 1000mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer the molecule until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

Depending on the type and severity of the disease, from about 0.001 mg/kg to abut 1000 mg/kg, more preferably about 0.01 mg to 100 mg/kg, more preferably about 0.010 to 20 mg/kg of the agonist antibody might be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs or the desired improvement in the patient's condition is achieved. However, other dosage regimes may also be useful.

EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

<u>EXAMPLE 1</u>

<u>Library Construction Of TPO Mimetic Sequences Grafted Into A Human Antibody</u>
<u>Framework</u>

An agonist TPO mimetic-peptide IEGPTLRQWLAARA (SEQ. ID. NO: 1) was grafted into the anti-tetanus toxoid (TT) Fab heavy chain CDR3 (HCDR3), replacing

5   the entire HCDR3 sequence GDTIFGVTMGYYAMDV (SEQ. ID. NO: 4). Figure 2A shows the sequence for the human tetanus toxoid antibody employed. Two grafting approaches were taken. In the first approach the agonist peptide was inserted into the H-CDR3 region with two glycines flanking each side. This was to reduce structural constraints on the grafted peptide so that it could more easily adopt the needed

10   conformation. In the second approach, two amino acid positions on each side of the peptide graft were randomized in order that the best presentation of the peptide could be achieved (Figure 3). These two approaches were taken in order to determine whether the peptide alone was sufficient or if specific residues were required for proper presentation of the agonist peptide on the antibody scaffold, thereby conferring

15   its activity to the antibody.

Figure 4 outlines the library construction process. Briefly, the anti-tetanus toxoid Fab was amplified as two fragments. Fragment A was amplified using a forward primer (N-Omp: 5' TAT CGC GAT TGC AGT GGC ACT GGC 3') (SEQ. ID. NO: 5) that annealed to the Omp A leader for the light chain in combination with a backward

20   primer (TPOCDR3-B: 5' GC CAG CCA TTG CCG CAG CGT CGG CCC TTC AAT YNN YNN TCT CGC ACA ATA ATA TAT GGC 3') (SEQ. ID. NO: 6) that annealed at the end of the heavy chain framework region (FR) 3. The reverse primer contained a tail encoding the new CDR3. Fragment B was generated using a forward primer (TPOCDR3-F: 5' CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG NNY

25   NNY TGG GGC CAA GGG ACC ACC GT 3')(SEQ. ID. NO:7) that annealed at the FR4 and the reverse primer Seq-G3Rev (5' TCA AAA TCA CCG GAA CCA GAG C 3') (SEQ. ID. NO: 8) which annealed in the gene III region of the plasmid, downstream of the heavy chain stop signal. The TPOCDR3-F primer also had a tail of bases that encoded the new CDR3 region. TAQ DNA Polymerase (Perkin Elmer) was used in the

30   following PCR program: 94° 30 seconds, then 30 cycles of 94° for 15 sec, 55° for 15

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seconds, and 72° for 90 seconds, followed by an extension period at 72° for 10 minutes, and a hold at 4°. After the fragments were generated by PCR and gel purified, they were combined for an overlap extension PCR. The new CDR3 primer encoded regions were complementary and provided 23 bases of overlap. Primers N-

5   Omp and SeqG3Rev were used in the overlap PCR protocol to generate the full Fab DNA product. Taq DNA Polymerase (Perkin Elmer) was used in the following PCR program: 94° 30", then 20 cycles of 94° 30", 56° 30", and 72° 3'15", then an extension period of 72° for 15' followed by a 4° hold. After gel purification of the Fab product, an Sfi 1 digest was performed at 50° for 5 hours. Inserts were ligated into Sfi 1 digested

10   pRL4 vector overnight. Ligation products were ethanol precipitated, resuspended in H$_2$O, and then electroporated into competent ER2537 bacteria (suppressor strain, New England Biolabs). Following one hour of shaking in 5 mls SOC, an equal volume of SB was added. Carbenicillin was added to 20ug/ml and the culture shaken for one hour at 37°, followed by one hour at 37° in 50ug/ml carbenicillin. The library culture was

15   transferred into a flask containing 100 mls fresh SB, 50 ug/ml Carbenicillin, and 10^{12} VCS M13 helper phage. After two hours at 37°, kanamycin was added to select for those bacteria that had been infected with helper phage. The following day, the overnight cultures were spun down and the phage in the supernate were precipitated on ice using 4% PEG/0.5 M NaCl. After spinning down the phage, the pellet was

20   resuspended in 1%BSA/PBS, filtered and dialyzed against PBS. Library phage were stored at 4°.

       The construction of Fabs containing the non-randomly linked peptide was performed as described above by substituting primers TPOCDR3-B and TPOCDR3-F with alternate specific primers. For PP-(IEGPTLRQWLAARA)-GG (SEQ. ID. NO: 25)

25   grafted antibody, primers used were TPOCDR3g-B (5' GC CAG CCA TTG CCG CAG CGT CGG CCC TTC AAT NGG NGG TCT CGC ACA ATA ATA TAT GGC 3') (SEQ. ID. NO: 9) and TPOCDR3g-F (5' CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG GGN GGN TGG GGC CAA GGG ACC ACC GT 3')(SEQ. ID. NO: 10). For GG-(IEGPTLRQWLAARA)-GG (SEQ. ID. NO: 29) grafted antibody, primers used were

30   TPO-CDR3-ggB (5' GC CAG CCA TTG CCG CAG CGT CGG CCC TTC AAT NCC NCC TCT CGC ACA ATA ATA TAT GGC 3')(SEQ. ID. NO: 11) and TPOCDR3g-F (5'

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CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG GGN GGN TGG GGC
CAA GGG ACC ACC GT 3') (SEQ. ID. NO: 12).

<u>Selection of the TPO Mimetic Peptide Heavy Chain CDR3 Library</u>

5   In order to select for the optimal peptide display, panning was performed on
human platelets. Because platelets express approximately 1800 TPO receptors per
cell on their surface (cMpl receptors), they represented a good cell target. In addition,
platelets are readily available from a local Blood Bank. To 1ml of concentrated indated
human platelets from the Blood Bank, 50 uls of freshly prepared Fab-phage were

10   added in a 15ml conical tube with 0.1% NaN$_3$. The tube was mixed at room
temperature for 1-2 hours. Typically, 10 mls of 50% human serum (taken off the
remaining platelets) + 50%{IMDM/10% FBS/0.1% azide/2mM EDTA} was added to the
phage/cells. Platelets were pelleted at 5500xg for 5 minutes at room temperature.
Supernatant was drained and the pellet was left resting under ~500 uls of the wash for

15   20 minutes. The platelets were very gently resuspended and then 10mls of 25%
human serum (taken off the remaining platelets) +75%{IMDM/10% FBS/0.1%
azide/2mM EDTA} was added to the phage/cells. The centrifugation, pellet rest, and
resuspension of the platelets was repeated. In panning rounds 3 and 4, a third wash
was performed. The washed phage/cells were transferred to an eppindorf tube and

20   spun at 5200xg. Phage were eluted from the platelets 10 minutes at room temperature
using acid elution buffer (0.1M HCl, 1mg/ml BSA, and glycine to pH 2.2). Platelets
were pelleted at max speed and the eluted phage transferred to a 50 ml conical tube,
neutralized with 2M Tris Base. Phage were then allowed to infect fresh ER2537
bacteria for 15 minutes at room temperature and amplified overnight as described

25   above. Four rounds of platelet panning were performed.
       After the fourth round of panning, pools of 3 Fab clones expressed as soluble
proteins in nonsuppressor bacterial strain TOP10F' (Invitrogen, Carlsbad, CA) were
tested by Facs for binding to platelets by utilizing the Fabs' HA epitope tag with rat
high affinity anti-HA followed by anti-Rat-FITC (Sigma, St. Louis, Missouri). 25 uls

30   indated concentrated human platelets (washed once with PBS / 5mM EDTA / 2% FBS)
were incubated with 100 uls bacterial supernate (60 uls bacterial supernate from three

pooled Fab clones were pre-incubated with 40 uls 5% Milk / PSS at 4° for 15 minutes) at room temperature for 20-30 minutes. 1 ml of FACS buffer (PBS / 2% FBS / 5mM EDTA) was added and cells spun down at 5200xg for 5 minutes. Pelleted cells were resuspended in 50 uls of l:10 diluted (in PBS / 1% BSA / 0.1% NaN$_3$) 2° anti-HA

5　antibody [Rat IgG anti-HA High Affinity clone 3F10 (Roche Molecular Biochemicals)] was added. After 30 minutes at room temperature, the cells were washed with 1 ml FACS buffer as above. Following centrifugation, cells were resuspended in 100 uls of 1:160 diluted (in PBS /1% BSA /0.1% NaN$_3$) 3° anti-Rat IgG-FITC antibody (Sigma) and incubated 20 minutes at room temperature in the dark. Cells were washed with 1

10　ml FACS buffer then resuspended in 200 uls FACS buffer for analysis. As a positive control a commercially available sheep anti-IIb/IIIa Ab followed by anti-sheep FITC was used. Many pools of Fabs were clearly positive for binding to platelets by Facs. Follow up Facs analysis was then performed to identify individual clones that bound to the platelets.

15

Examination Of Individual Candidates By Binding Activity

Several Fabs, as bacterial supernatants, were tested for reactivity to the original antigen tetanus toxoid in order to determine if that binding specificity was retained. The antibody scaffold anti-TT Fab does bind to its antigen TT, but not to BSA. However,

20　four TPO-mimetic peptide grafted Fab clones did not show significant binding to TT or BSA. As seen in previous experiments, the replacement of anti-TT Fab HCDR3 was sufficient to change the specificity of the antibody.

To further examine the binding capabilities of Fabs, Facs analysis was performed on CMK cells, a Megakaryocytic cell line (from German Collection of

25　Microorganisms and Cell Cultures) which also expresses the cMpl receptor. Fab clones that bound CMK cells were then analyzed to verify that the platelet and CMK cell binding was occurring via the cMpl receptor. For that experiment, 293 EBNA cells were transfected with or without the cMpl-R, which had been cloned from Tf-1 cells by RT-PCR. 1 X 10^6 transfected cells were incubated with bacterial supernate from each

30　Fab clone (pre-blocked as described above) for 20-30 minutes at room temperature. Cells were spun down at 2000 rpm for 5 minutes. Pelleted cells were resuspended in

90 uls FACS buffer (PBS / 2% FBS / 1mM EDTA) then 10 uls of 2° anti-HA antibody [Rat IgG anti-HA High Affinity clone 3F10 (Boehringer Mannheim Biochemicals) was added for a final 1:10 dilution. After 20 minutes at room temperature, the cells were washed with 1 ml FACS buffer. Following centrifugation, cells were resuspended in

5   100 uls of 1:50 diluted (in PBS /1% BSA /0.1% NaN₃) 3° anti-Rat IgG-PE antibody (Research Diagnostics Incorporated, RDI) and incubated 20 minutes at room temperature in the dark. Cells were washed with 1 ml FACS buffer then resuspended in 200 uls FACS buffer for analysis. Fabs selected during panning demonstrated strong binding to cells transfected with the cMpl-R but not to control vector transfected

10   cells lacking the cMpl-R. This indicates that cell surface binding was occurring specifically through the cMpl receptor. Anti-TT Fab does not bind to control vector or cMpl-R transfected 293 cells. However, Fab clone X1c shows a shift from 3% binding of control (non-cMpl receptor) transfected cells to 95% binding of cells expressing the cMpl-R.

15   <u>Examination Of Individual Candidates By Sequence</u>

Sequence analysis of Fab clones which specifically bound to the cMpl receptor (see Fig. 5), revealed the selection of preferred amino acids at the downstream linkage site. The DNA sequence data was analyzed and the amino acid and DNA sequences are as follows:

Clone	Binding Properties	SEQ. ID. NO	Sequence
X1a	weak	25	Pro Pro (14 aa peptide) Gly Gly
X1a-11	weak	27	Gly Gly (14 aa peptide) Gly Gly
X1a-13	weak	29	Gly Gly (14 aa peptide) Gly Gly
X1c	strong	31	Trp Leu (14 aa peptide) Pro Val
X2c	weak	33	Met Ile (14 aa peptide*) Val Gly
X3a	strong	35	Val Val (14 aa peptide) Pro Val
X3b	strong	37	Gly Pro (14 aa peptide) Pro Asp
X4b	strong	39	Leu Pro (14 aa peptide) Pro Val
X4c	strong	41	Ser Leu (14 aa peptide) Pro Ile
X5a	strong	43	Thr Met (14 aa peptide) Pro Val
X5c	strong	45	Trp Leu (14 aa peptide) Pro-Val
X7a	weak	47	Thr Arg (14 aa peptide*) Cys Ser
X7b	weak		deletion mutant this clone has lost the peptide
X7c	strong	49	Gln Thr (14 aa peptide) Pro Asp

All clones which demonstrated strong binding, were found to contain a proline just downstream of the 14 amino acid TPO mimetic peptide. Selection by panning of a proline in the downstream linker position represents determination of a surprising amino acid choice which confers improved binding characteristics to the grafted TPO mimetic peptide. Weak binders did not contain this proline although they still contained the TPO mimetic peptide. It should be noted that clone X7a had a silent mutation in this peptide (GCG to GCA retaining the Ala at position 11 in the peptide) and that

clone X2c had a mutation in this peptide switching a Thr for Ala at position 11 of the peptide.

Biological Assays

Clones were tested for agonist activity using a transcriptional based assay measuring luciferase activity driven by the c-Fos promoter. Dimerization of the cMpl receptor activates Jak which stimulates the MAP kinase pathway. Thus activation can be measured by assaying luciferase production and activity stimulated by MAP kinase via the cFos promoter. Since dimerization of the cMpl receptor is required for activation, either full IgG or dimerized Fab fragments capable of dimerizing the receptor, could be used to stimulate cMpl receptor activity. Fabs produced in bacteria were dimerized via the HA tag utilizing the 12CA5 anti-HA antibody. Increasing amounts of 12CA5 was added to the bacterial Fabs to dimerize the Fab clones in order that they might in turn dimerize and activate the cMpl receptor. For measurement of agonist activities Fab containing bacterial supernatants (2mls) mixed with 12CA5 were applied to NIH3T3 cells which had been co-transfected with either a control vector or the cMpl receptor and the Fos promoter/luciferase reporter construct. Co-transfections of 3T3 cells were performed by plating NIH 3T3 cells at 3×10^5 cells per 6 cm dish and then transfecting the following day. NIH 3T3 cells were transfected using the Effectine lipofection reagent (Qiagen), transfecting each plate with 0.1 ug pEGFP (a tracer to measure transfection efficiency), 0.2 ug of the Fos promoter/luciferase construct and 0.7 ug of either the empty control vector or the plasmid expressing the cMpl receptor. 3T3 cells were placed in 0.5% serum 24 hours post transfection and incubated for an additional 24 hours in this low serum media to reduce the background activation of the Fos promoter. Antibody supernatants were then applied to these cells for 6 hours. Cells were harvested and luciferase assays performed using 50 ug of cell lysate. No activation was stimulated by the antibodies in the absence of cMpl receptor expression. However, agonist activity was observed in cMpl receptor co-transfected 3T3 cells. This allowed us to demonstrate that the agonist activity observed was through the cMpl receptor and its interaction with the antibody. The data is as follows:

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Bacterial Supernatants with 12CA5		
CELL Treatment	Relative Fold Luciferase activity	
	CMpl-R transfected	Control transfected
Untreated:	1.0	1.0
+ 10% FCS	3.43	3.0
+ TPA	2.3	2.42
+ TPO	2.32	0.76
+ X4c Sup (alone)	1.03	0.94
+ X4c + 60ul α-HA	1.97	0.84
+ X4c + 30ul α-HA	1.52	0.87
+ X4c + 10ul α-HA	1.22	0.86
+ X4c + 3ul α-HA	0.94	0.68
+ X4c + 1ul α-HA	0.91	1.05
+ X4c + 0.3ul α-HA	1.01	0.95

Activation of cMpl receptor can be tested in a similar manner using full IgGs (converted from Fab as described herein) produced by transient or stable transfection of mammalian cells rather than bacterially produced Fabs dimerized by anti-HA 12CA5. Experimentally transient transfection can be performed essentially as described here. For transfections 2×10^6 cells (such as 293 EBNA) would be plated in 6cm dishes for each test sample. The following day each plate would be transfected with 2.5 ug of total DNA (2 ug total of the light chain and heavy chain plasmid(s), 0.25 ug of pAdVAntage (Promega, Madison, Wisconsin), and 0.25 ug of pEGFP) using the

Effectine reagent (Qiagen). The 293 cells would be placed in 0.5% serum 24 hours
post transfection and incubated for an additional 24 hours in this low serum media to
obtain full IgG. Residual growth factors are negligible in this media in stimulating
receptors as seen in controls experiments. After 24 hours supernatants would be
collected and spun for 5 minutes at 3000rpm to remove any residual cells. For
measurement of agonist activities of the full IgGs, 3mls of the conditioned 293 cell
supernatants would be applied to NIH3T3 cells as described above.

<div align="center">EXAMPLE 2</div>

Additional Libraries Containing TPO Mimetic Sequences Grafted Into A Human
Antibody Framework

Another approach to linking two agonistic peptides together in an antibody
framework is to insert the agonist peptide in more than one position within a single Fab
fragment. In order to do that, additional libraries containing TPO mimetic sequences
grafted into a human antibody framework were constructed. Following selection of
peptides properly presented in the context of CDR1, CDR2 or CDR3 of the light chain,
or CDR2 of the heavy chain, the binding sequences were combined into a single Fab
molecule, for example as listed in Table 1 below, and analyzed for enhanced activity.

<div align="center">

TABLE 1

</div>

Peptide 1	Peptide 2
H-CDR3	H-CDR2
H-CDR3	L-CDR1
H-CDR3	L-CDR2
H-CDR3	L-CDR3
H-CDR2	L-CDR2
H-CDR2	L-CDR3
L-CDR3	L-CDR2

Four additional libraries have been constructed separately replacing the heavy
chain CDR2 as well as the light chain CDR1, CDR2 and CDR3 with the TPO mimetic
peptide flanked by 2 random amino acids using an NNK doping strategy. The
generation of the libraries was similar to that described for the heavy chain CDR3 TPO

<div align="center">48</div>

peptide library, except that only the chain (heavy or light) being modified to form a library was amplified by PCR and used as the insert. PCR was performed using Expand High Fidelity PCR System (Roche) which contains a mixture of Taq and Pwo Polymerases. The first round of PCR was performed using the program: 94° 30", then

5 30 cycles of 94° 15", 56° 30", and 72° 2', followed by elongation for 10' at 72° and a 4° hold. Overlap PCR was performed for 10 cycles without primers using the program listed above to allow the full DNA template to be generated by the polymerases. Primers were then added to the PCR reaction tubes for 20 cycles of the same program for amplification.

10   For the HCDR2 library, the fragment A was created using the forward primer lead VH (5' GCT GCC CAA CCA GCC ATG GCC 3')(SEQ. ID. NO: 13), which annealed at the pel B leader signal located in front of the heavy chain, and the reverse primer HR2 CMPL ANTI (5' AGC CAG CCA CTG GCG CAG GGT TGG GCC TTC GAT MNN MNN TCC CAT CCA CTC AAG CCC TTG 3')(SEQ. ID. NO: 51) that

15 annealed at the end of the heavy chain FR2. The reverse primer contained a tail encoding the new CDR2. Fragment B was created using forward primer HR2 cMpl CODE (5' CCA ACC CTG CGC CAG TGG CTG GCT GCT CGC GCT NNK NNK AGA GTC ACC ATT ACC GCG GAC 3')(SEQ. ID. NO: 14) which annealed at FR3 of the heavy chain and reverse primer N-dp (5' AGC GTA GTC CGG AAC GTC GTA CGG

20 3')(SEQ. ID. NO: 15) which annealed in the HA epitope tag region of the plasmid, downstream of the heavy chain constant region. The HR2 cMpl CODE primer also had a tail of bases that encoded the new CDR2 region. After the fragments were generated by PCR and gel purified, they were combined for an overlap extension PCR. The new CDR2 primer encoded regions were complementary and provided 24 bases of

25 overlap. Primers leadVH and N-dp were used in the overlap PCR protocol to generate the full heavy chain DNA product. Following gel purification of the heavy chain product, a Xho I / Spe I digest was performed at 37° for 3 hours. Inserts were gel purified and then ligated into Xho I / Spe I digested pRL4 vector containing the anti-TT light chain. Ligation products were precipitated and electroporated into ER2537 bacteria as

30 described above for the generation of the Fab-phage library.

The light chain CDR3 library was similarly made using primers for Fragment A of forward primer N-omp and reverse primer LR3 cMpl ANTI (5' AGC CAG CCA CTG GCG CAG GGT TGG GCC TTC GAT MNN MNN ACA GTA GTA CAC TGC AAA ATC 3') (SEQ. ID. NO: 16) and for Fragment B of forward primer LR3 cMpl CODE (5' CCA ACC CTG CGC CAG TGG CTG GCT GCT CGC GCT NNK NNK TTC GGC CAA GGG ACC AAG GTG 3')(SEQ. ID. NO: 17) and reverse primer leadB (5' GGC CAT GGC TGG TTG GGC AGC 3') (SEQ. ID. NO: 18). Primer leadB annealed to the pelB leader sequence located before the VH. The LR3 cMpl ANTI reverse and LR3 cMpl CODE forward primers annealed to the FR3 and FR4 of anti-TT light chain respectively. Both LR3 cMpl primers contain a tail of nucleotides encoding the new CDR3 peptide library, which provides the 24 basepair overlap region for the fusion PCR of Fragment A and Fragment B. Following purification of the light chain PCR products, a Sac I / Xba I digest was performed at 37° for 3 hours. The light chain fragments were then ligated into Sac I / Xba I digested pRL4 containing the anti-TT heavy chain overnight at room temperature. Ligation products were precipitated and electroporated into ER2537 bacteria as described above for the generation of the Fab-phage library.

The construction of the light chain CDR2 library was carried out as described above for the light chain CDR3 library with the exception that specific primers LR2 cMpl ANTI (5' AGC CAG CCA CTG GCG CAG GGT TGG GCC TTC GAT MNN MNN ATA GAT GAG GAG.CCT GGG AGC 3')(SEQ. ID. NO: 19) which annealed at the end of light chain FR2 and primer LR2 cMpl CODE (5' CCA ACC CTG CGC CAG TGG CTG GCT GCT CGC GCT NNK NNK GGC ATC CCA GAC AGG TTC AGT 3')(SEQ. ID.

NO: 20) which annealed at the beginning of light chain FR3 were used in place of the LR3 cMpl primers.

The construction of the light chain CDR1 library was also carried out as previously described for the light chain CDR3 library with the exception that specific primers TPOLR1CODE (5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTNNKNNKTGGTACCAGCAGA AACCTGGC 3') (SEQ. ID. NO: 58) which annealed at the beginning of the light chain

FR2 and the primer TPOLR1ANTI
(5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATMNNMNNGCAGGAGAGGGT
GGCTCTTTC 3') (SEQ. ID. NO: 59) which annealed to the end of the light chain FR1
were used in place of the LR3 cMpl primers.

5   The three additional libraries, which separately replace the heavy chain CDR2
and the light chain CDR2 and CDR3 with the TPO mimetic peptide flanked by 2
random amino acids using the NNK doping strategy, were separately panned on
platelets, as was previously described in Example I for the heavy chain CDR3
replacement library. Four rounds of panning were performed and clones were
10   screened by FACS on platelets and cMpl receptor transfected 293 cells as previously
described. Two positive clones were obtained from these screens. These clones had
the TPO mimetic peptide in the heavy chain CDR2. Unlike with the heavy chain CDR3
clones neither of the heavy chain CDR2 clones had a proline in downstream position.
Instead both were found to contain a tyrosine in the upstream position (See Figure 9-
15   clones
HC-CDR2 No. 24 and No. 39).

      The libraries, including LCDR1, were separately subjected to another panning
experiment using cMpl receptor transfected 293 cells instead of platelets during the
panning. The 293 cells were observed to reproducibly transfect at a high efficiency
20   and express very high levels of the functional cMpl-receptor on their surface. Thus
these cells represented a good cell target for use in panning. For these experiments
different groups of plates of 293 cells were separately and sequentially transfected
four days in a row. Each group of plates was then sequentially used for the four
separate rounds of panning. Each round of harvesting of the cells and panning
25   occurred two days after transfection. For harvesting, cells were removed from the
plates using cell disassociation buffer, spun down at 1500 rpm for 5 minutes and re-
suspended in IMDM supplemented with 10% FCS, 0.1% sodium azide and 5mM
EDTA at a concentration of 1 x 10^6 cell per ml (3×10^6 for LC-CDR1). In the round one
pan, 3 x 10^{11} phage from each library were separately applied to 2 ml of cells (6×10^6
30   for LC-CDR1 and 2 x 10^6 cells for all others) and rotated in a 15 ml conical tube for two
hours at room temperature. Cells were washed twice using 10 mls of the IMDM/10%

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FCS/0.1% sodium azide/5mM EDTA buffer. Phage were eluted in acid and amplified as previously described in Example 1. In round two 4 x 10^6 cells ($6x10^6$ for LC-CDR1) were used in 2 ml of buffer and 3 x 10^{11} phage from the amplified round one eluted phage was combined with 3 x 10^{11} phage from the un-panned library and added to the cells. Washing, elution and amplification proceeded similar to round one. In round three 4 x 10^6 cells ($6x10^6$ for LC-CDR1) were used in 2 ml of buffer and 3 x 10^{11} phage from the amplified round two eluted phage were used. Cells were washed three times prior to elution. In round four, 4 x 10^6 cells ($6x10^6$ for LC-CDR1) were again used in 2 ml of buffer and 3 x 10^{11} phage from the amplified round three eluted and amplified phage were used. Cells were again washed three times prior to elution. At least thirty individual clones were screened by FACS on cMpl receptor transfected 293 cells as previously described. 12 positive clones were obtained from the heavy chain CDR2 library and 25 positive clones were obtained from the light chain CDR2 library, and 14 positive clones were obtained from the light chain CDR1 library. Clones were further analyzed by DNA sequence. The selected flanking amino acid residues for the positive clones are depicted in the attached Figure 9. It is of interest to note that the light chain CDR2 grafted Fabs have a strong selection for a proline (Pro) upstream of the TPO mimetic peptide.

<div align="center">EXAMPLE 3</div>

Combinations of the TPO mimetic peptide grafted Fab clones from Figure 9 have been generated. Thus a single antibody might contain multiple copies of the TPO mimetic peptide within a single light or heavy chain. Alternatively, both the light and heavy chains might contain peptide grafts giving multiple copies within a single Fab. Positive clones selected from the same CDR library were pooled. New libraries were constructed by combining the pool of one TPO mimetic peptide containing CDR with the pool of another. Combinations where one of the TPO mimetic peptides is in the light chain and the other is in the heavy chain are made using simple cloning techniques using the pooled plasmid DNAs, and the unique restriction sites flanking the heavy (Xho I-SpeI) and light chains (Sac I-Xba I). For example, the plasmid DNA for the H-CDR3 peptide grafted heavy chains were combined and digested by Xho I

<div align="center">52</div>

and Spe I. The purified heavy chain inserts were ligated into the Xho I/Spe I digested plasmid containing the L-CDR2 grafts. The resulting library contained heavy chains with CDR3 peptide grafts and light chains with CDR2 peptide grafts. It should be understood that individual clones could also be combined rather that using pools of

5   clones for the pairing of two peptide containing CDRs. For example, a single heavy chain clone with a CDR3 peptide graft was paired with several individual light chain CDR1 clones to create Fabs with multiple copies of TPO mimetic peptides.

Combinations where two TPO mimetic peptides were combined within a given heavy chain was performed using overlap PCR to generate the fragment for cloning.

10   Two overlapping primers which bind between CDR2 and CDR3, and flanking primers, such as "N omp" and "lead B" primers from the light chain and "Lead VH" and "Ndp" primers for the heavy chain were used. For example, to combine H-CDR2 and H-CDR3 the first PCR was performed using lead VH (a primer that anneals in the vector at the heavy chain pelB leader signal) and a reverse primer annealing at FR3 using

15   the H-CDR2 pooled plasmid DNA as the template. The sequence of that primer was 5' CCA TGT AGG CTG TGC CCG TGG ATT 3' (SEQ. ID. NO: 63). In a separate reaction, the pooled plasmids containing the H-CDR3 grafts underwent PCR with a forward primer annealing in FR3 (which is complementary to the above FR3- reverse primer) and N-dp (which anneals in the vector at an epitope tag sequence). The

20   sequence of that primer was 5' CCA CGG GCA CAG CCT ACA TGG AGC 3' (SEQ. ID. NO: 64). The first PCR products were purified then combined in an overlap PCR reaction, where fusion of the two fragments occurred through the complementary FR2 sequences. The full heavy chain product was cloned by Xho I/Spe I into a plasmid containing the anti-tetanus toxoid light chain. In all, five classes of Fabs

25   bearing multiple copies of TPO mimetic peptides were created as detailed in Table 2 below.

TABLE 2

Peptide 1	Peptide 2	Peptide 3
H-CDR3	H-CDR2	
H-CDR3	L-CDR2	
H-CDR2	L-CDR2	
H-CDR3	H-CDR2	L-CDR2
H-CDR3	L-CDR1	

20 clones from each combination library and four individual clones of H-CDR3 plus L-CDR1 were tested for biological activity in a luciferase reporter assay as previously described. See Figures 10 and 11. In both experiments, the negative controls can include uninduced cells, cells treated with an irrelevant Fab (anti-tetanus toxoid), cells treated with a Fab clone that only weakly binds cMpl receptor, and X4b and/or X1c Fabs which do bind the cMpl receptor but only have a single binding domain and so can not activate the receptor. The positive control was the addition of TPO. All remaining samples were from the newly formed combination libraries. As can be observed, several clones have significant activity as Fabs. This indicates that incorporation of multiple TPO mimetic peptides into a single Fab molecule is able to bind two receptors and cause receptor activation.

In fact, using Fab 59 the agonistic activity obtained can be as high as native TPO activity. Clone 59 containing two TPO mimetic peptides (HC CDR3 sample x4c and LC CDR2 sample 19 as identified in Fig. 9) and a His6 tag was partially purified from a bacterial periplasmic prep by FPLC using nickel column chromatography. The activity of this Fab was measured and found to be approximately equivalent to that of TPO (see Figure 12), as estimated by cMpl-R specific induction of luciferase activity in direct comparison to known concentrations of TPO. A quantitative western blot was performed in order to determine the Fab 59 antibody concentrations.

These Fabs, or various other two CDR combinations, could be used as a therapeutic product. Alternatively, these clones could be converted to framework germline sequences (either with or without codon optimization) for use as a therapeutic agent so long as activity was maintained.

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EXAMPLE 4

The TPO mimetic peptide graft in Fab clone X4b has been transplanted into the heavy chain CDR3 of another antibody framework, 5G1.1. Construction of 5G1.1 is described in U.S. Appln. Ser. No. 08/487,283, incorporated herein by reference. The

5   sequence was cloned into 5G1.1 in such a fashion as to replace the native CDR3 with 5' ttg cca ATT GAA GGG CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG cct gtt 3' (SEQ. ID. NO: 65). The peptide graft translated into amino acids is Leu Pro Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Pro Val (SEQ. ID. NO: 66). The 5G1+peptide was produced as a whole IgG antibody (See Figures 13A and 13B).

10   Purified 5G1.1+peptide antibody as well as the parental 5G1.1 were analyzed for their ability to bind to cMpl receptor by FACS analysis. Binding to receptor expressing and non-receptor expressing 293 cells was compared. See Figure 14. The FACS staining was performed essentially as described previously herein, with the exception that the detection was done using PE conjugated F(ab')2 fragment of goat

15   anti-human IgG (H+L). The negative controls of 3° only anti-tetanus toxoid irrelevant Fab, and Fab X1a which binds weakly to cMpl receptor all showed very little staining. However, binding Fabs X1c and X4b showed strong staining as did the 5G1.1+peptide. None of those clones demonstrated binding to the non-receptor expressing cells indicating that the cell staining is occurring through specific

20   recognition of the cMpl receptor. The parental 5G1.1 without the TPO mimetic peptide did not show staining to any of the cells tested.

The ability of the 5G1.1+ peptide whole IgG to activate the cMpl receptor using the luciferase reporter assay has been determined (see Figure 15). The results herein indicate that the configuration of a whole IgG causes steric limitations in its ability to

25   productively bring the two cMpl receptors together for activation. The activity of the 5G1.1 full IgG construct containing the TPO mimetic peptide in the heavy chain CDR3 positions, was only weakly activating and required approximately 100-200 fold higher molar concentrations as compared to TPO, to stimulate equivalent activity. As was previously observed with the binding experiments, activation by the 5G1.1 containing

30   the peptide was observed only when the cMpl-R was expressed on the cell surface.

No receptor specific binding or activity was observed with the parental 5G1.1 not containing the peptide. These results demonstrate that binding and activity of the TPO mimetic peptide and selected amino acid flanking sequences is not limited to or specific for the Tetanus Toxoid antibody framework, but can be applied to other antibody frameworks. Thus the flanking amino acid sequences that were selected during panning are specific for presentation of the TPO mimetic peptide within a given CDR position, but not for amino acid sequence of the antibody framework.

EXAMPLE 5

Library Construction Of Epo Mimetic Sequences Grafted Into A Human Antibody Framework

An agonist EPO mimetic-peptide DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 3) (designated EMP2 in Wrighton et al. 1996) was grafted separately into the anti-tetanus toxoid Fab heavy and light chain CDR3 region creating two antibody libraries as XXDYHXRMGPLTWVXKPLGGXX (SEQ. ID. NO: 71). Randomized positions were generated using an NNK doping strategy. As with the TPO mimetic peptide, two amino acids flanking the EPO mimetic peptide were randomized in order to select for the optimum presentation of the peptide. In addition the cysteine residues, which formed a disulfide bridge in the original cyclic peptide, were randomized. This was done not only because the CDRs already form loop structures and so the disulfide bridge was not necessary to constrain the peptide, but also because the cysteines might in fact disrupt the normal disulfide bonds of the antibody.

The CDR 3 of the anti-TT antibody heavy chain was completely replaced by the EPO peptide library graft. The generation of the library was essentially as described for the TPO heavy chain CDR2 library. Two alternate primers were used for the HCDR3 library: the reverse primer HR3 EPO ANTI (5' CAC CCA GGT CAG TGG GCC CAT GCG MNN ATG ATA GTC MNN MNN TCT CGC ACA ATA ATA TAT GGC 3')(SEQ. ID. NO: 21) that annealed at the end of the heavy chain FR3, and forward primer HR3 EPO CODE (5' CGC ATG GGC CCA CTG ACC TGG GTG NNK AAA CCA CTG NNK NNK TGG GGC CAA GGG ACC ACG GTC 3')(SEQ. ID. NO: 22) which annealed at FR4 of the heavy chain.

The light chain CDR3 EPO peptide library was constructed essentially as described above for the light chain CDR3 TPO peptide library using reverse primer LR3 EPO ANTI (5' CAC CCA GGT CAG TGG GCC CAT GCG MNN ATG ATA GTC MNN MNN ACA GTA GTA CAC TGC AAA ATC 3') (SEQ. ID. NO: 23) that annealed at

5   the end of light chain FR3 and forward primer LR3 EPO CODE (5' CGC ATG GGC CCA CTG ACC TGG GTG NNK AAA CCA CTG NNK NNK TTC GGC CAA GGG ACC AAG GTG 3') (SEQ. ID. NO: 24) which annealed to FR4 of the light chain.

Selection Of The Epo Mimetic Peptide Heavy Chain Cdr3 Library And Light Chain Cdr3 Library

10   Selection for peptide presentation was performed by solid phase panning on the soluble EPO receptor. In this method, 1ug of purified human EPO-soluble R (hEPO-sR from R&D Systems, Minneapolis, MN cat#307-ER-050) was immobilized on a microtiter dish overnight at 4°. After washing off free hEPO-sR, the plates were blocked with 1% BSA/PBS for one hour at 37°. Phage, prepared as described above,

15   were added to the wells and incubated two hours at 37°. Washes were performed using PBS/0.5% Tween 20 for 5' at room temperature per wash. 1, 5, 10, and 10 washes were performed in the first, second, third and fourth panning rounds respectively. After the washing steps were completed, the bound Fab-phage were eluted with 30 uls of elution buffer for 10' at room temperature. Eluted phage were then

20   neutralized and amplified as described in Example 1.

EXAMPLE 6

A library was generated by the insertion of a TPO mimetic peptide and previously selected flanking amino acids (NP-IEGPTLRQWLAARA-RG) (SEQ. ID. NO: 61) into a collection of human kappa gene fragments, in this case the CDR2 of the

25   light chain. Stocks of human kappa light chains from multiple human peripheral blood lymphocyte (PBL) donors had been previously generated and cloned into pBluescript II SK+. Those constructs served as the source of antibody gene fragments.

Antibody Gene Banks

Total RNA from human PBLs was isolated using TRI Reagent (Molecular
Research Center, Cincinnati, OH) followed by mRNA purification with Oligotex mRNA
purification System (QIAGEN, Valencia, CA) according to kit instructions. First strand
cDNA was made using SuperScript RTase II cDNA Synthesis Kit (Life Technologies,
Rockville, Maryland) with a modified oligo dT primer. The sequence of the primer was
5' TAGGATGCGGCCGCACAGGTC(T_{20}) 3' (SEQ. ID. NO: 62). Samples were
cleaned up over a PCR purification Kit spin column (QIAGEN, Valencia, CA) according
to kit directions. Light chain products were amplified using the reverse "Not I" primer
and forward primers which annealed at the framework 1 (FR1) position of Kappa
chains on the 1st strand cDNA. The "Not I" primer had sequence which was identical
to the 5' end of the modified oligo dT primer (5' TAGGATGCGGCCGCACAGGTC
3')(SEQ. ID. NO: 72). The set of Kappa FR1 primers used were:

XVB Vk1a   CACGCGCACAACACGTCTAGARACATCCAGATGACCCAG (SEQ. ID.
NO: 73)

XVB Vk1b   CACGCGCACAACACGTCTAGAGMCATCCAGTTGACCCAG (SEQ. ID.
NO: 74)

XVB Vk1c   CACGCGCACAACACGTCTAGAGCCATCCRGATGACCCAG (SEQ. ID.
NO: 75)

XVB Vk1d   CACGCGCACAACACGTCTAGAGTCATCTGGATGACCCAG (SEQ. ID.
NO: 76)

XVB Vk2a   CACGCGCACAACACGTCTAGAGATATTGTGATGACCCAG (SEQ. ID.
NO: 77)

XVB Vk2b   CACGCGCACAACACGTCTAGAGATRTTGTGATGACTCAG (SEQ. ID.
NO: 78)

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XVB Vk3a   CACGCGCACAACACGTCTAGAGAAATTGTGTTGACRCAG   (SEQ. ID.
NO: 79)

XVB Vk3b   CACGCGCACAACACGTCTAGAGAAATAGTGATGACGCAG   (SEQ. ID.
NO: 80)

5   XVB Vk3c   CACGCGCACAACACGTCTAGAGAAATTGTAATGACACAG   (SEQ. ID.
NO: 81)

XVB Vk4a   CACGCGCACAACACGTCTAGAGACATCGTGATGACCCAG   (SEQ. ID.
NO: 82)

XVB Vk5a   CACGCGCACAACACGTCTAGAGAAACGACACTCACGCAG   (SEQ. ID.
10   NO: 83)

XVB Vk6a   CACGCGCACAACACGTCTAGAGAAATTGTGCTGACTCAG   (SEQ. ID.
NO: 84)

XVB Vk6b   CACGCGCACAACACGTCTAGAGATGTTGTGATGACACAG   (SEQ. ID.
NO: 85)

15   A typical amplification reaction contained 2 μls cDNA reaction, dNTPs, "Not I"
reverse primer, one of the XVB forward primers, Opti-prime buffer #5 (Stratagene, La
Jolla, CA), and Expand High Fidelity polymerase mixture (Roche Molecular
Biochemicals, Indianapolis, IN). Samples were heated to 94°C for 2 minutes, then
carried through 10 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 1
20   minute, followed by 20 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C
for (1 minute + 5 seconds/cycle). The cycles were followed by an extended incubation
at 72°C (7 minutes) prior to 4°C hold. Products were ethanol precipitated and then gel
purified. Fragments of approximately 850bp were isolated and then digested with Xba
I and Sac I. The resulting kappa products were ligated into pBluescript II SK+ that had
25   likewise been digested with Xba I and Sac I. The ligation products were
electroporated into Top10F' (Invitrogen, Carlsbad, CA) and grown overnight. The
bacterial pellet was used to isolate the Kappa library DNA with QIAGEN's MAXIprep
DNA isolation kit.

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Construction Of Framework Library With TPO Mimetic Peptide

       For construction of the TPO light chain framework library, equal amounts of four different kappa light chain libraries from four different patients were used as the starting template for the PCR reactions (25ng total per reaction). The TPO mimetic

5   peptide and selected flanking amino acids were incorporated into the light chains by overlap PCR. In the first round of PCR a set of reverse primers (VK ANTI primers) which bound to the kappa light chain FR2 were separately combined with the forward T7 seq-F primer (5'-ATTAATACGACTCACTATAGGG-3') (SEQ. ID. NO: 86) to synthesize the N terminal piece of the light chain and part of the TPO mimetic peptide

10   within the LC CDR2 position. A second set of forward primers (VK CODE primers), which bound to FR3, were combined separately with the T3 reverse primer (5'-AATTAACCCTCACTAAAGGG-3') (SEQ. ID. NO: 87) to synthesize the rest of the TPO mimetic peptide within the LC CDR2 position and the C terminal half of the light chain by PCR. Separate reactions were performed for each pair of primer combinations in

15   duplicate.

VK6ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTCCTGATGAGGAGC
TTTGGRG-3' (SEQ. ID. NO: 88)

VK5ANTI
20   5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTTTGAATAATGAAAA
TAGCAG-3' (SEQ. ID. NO: 89)

VK4ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTGTAAATGAGCARCT
TAGGAG-3' (SEQ. ID. NO: 90)

25   VK3ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATGAGGAGC
CTGGGMG-3' (SEQ. ID. NO: 91)

VK2AANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAAATTAGGCGCC
30   TTGGAG-3' (SEQ. ID. NO: 92)

60

VK2BANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATYAGGAGCT
GTGGAG-3' (SEQ. ID. NO: 93)

VK1AANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATCAGGAGCT
TAGGA-3' (SEQ. ID. NO: 94)

VK1BANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTRTAGATCAGGAGCT
TAGGG-3' (SEQ. ID. NO: 95)

VK1CANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATCAGGGACT
TAGGG-3' (SEQ. ID. NO: 96)

VK1DANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATCAGGYGCT
TAGGG-3' (SEQ. ID. NO: 97)

VK6CODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCCTCGA
GGTTCAG-3' (SEQ. ID. NO: 98)

VK5CODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGAATCCCACCTC
GATTCAG-3' (SEQ. ID. NO: 99)

VK4CODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCTGACC
GATTCAG-3' (SEQ. ID. NO: 100)

VK3ACODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGCATCCCAGMCA
GGTTCAG-3' (SEQ. ID. NO: 101)

VK3BCODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGTATCCCAGCCA
GGTTCAG-3' (SEQ. ID. NO: 102)

VK2ACODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGAGTSCCAGAYA
GGTTCAG-3' (SEQ. ID. NO: 103)

VK2BCODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCWGACA
GRTTCAG-3' (SEQ. ID. NO: 104)

VK1ACODE
5   5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCATCAA
GGTTCAG-3' (SEQ. ID. NO: 105)

VK1BCODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCATCTC
GGTTCAG-3' (SEQ. ID. NO: 106)

10   Fragments from the first rounds of PCR were gel purified. Those purified

fragments were then combined, in an antibody family specific manner, in overlap PCR

reactions to generate the full light chain. Reactions for each family were performed in

triplicate using 40ng of both the N-terminal and C-terminal piece of the light chain in

each reaction. The reactions were run for 10 cycles prior to the addition of the T3 and

15   T7 Seq-F primers, followed by an additional 25 cycles after primer addition. The full

length LC fusion PCR products were gel purified, digested with Sac I and Xba I, and

then again gel purified. The light chain inserts were then ligated into an appropriate

phage display vector, which had been similarly digested with Xba I and Sac I and gel

purified. The pRL5-kappa vector used had restriction sites which were compatible with

20   the LC fragments and contained the remaining Kappa constant region from the native

Sac I site to the C-terminal Cys. In addition, the anti-tetanus toxoid heavy chain was

inserted into the vector by Xho I and Spe I for Fab production.

            The ligation mixture was transformed by electroporation into XL-1 Blue bacteria

(Stratagene, La Jolla CA) and amplified. The library was panned four rounds on 293

25   EBNA cells transfected with the cMpl-R in a manner similar to that previously

described. Clones obtained during panning were screened for binding by FACs

analysis on 293 EBNA cells transfected with or without the cMpl-R as previously

described. A number of clones, which specifically bound the cMpl-R, were obtained.

DNA fingerprinting of the resulting light chains by digestion with Bst N1 indicated that

30   the clones could be divided into 5 different groups. Partial sequencing of 8 of these

clones showed that frameworks from at least two different kappa light chain families

were selected during the panning (VK1 and VK3). In an initial test, 3 of the light chain

framework clones were combined with the heavy chain of X4b to create a Fab with 2 TPO mimetic peptides. These clones induced activation of the cMpl-R in luciferase assays as previously described. The level of activation using bacterial supernatants of one such clone 429/X4b (see Figure 16) was approximately 10-20 fold lower than that observed with TPO, as estimated by comparing activity to known concentrations of TPO and using quantitative western blots to determine the concentration of the antibody in the supernatant. Additional clones can be screened in a similar fashion on order to identify clones with greater activity.

These Fabs, or various other LC, HC or intrachain CDR combinations, could be used as a therapeutic product. Alternatively, these clones could be converted to framework germline sequences (either with or without codon optimization) for use as a therapeutic agent so long as activity was maintained.

Modification of the Phage Display Vectors pAX131 and pRL5

The Not I site in pAX131 was removed by digesting the vector with Not I, using Klenow polymerase to fill in the Not I overhangs, and then re-ligating the vector. See Figure 17. Further modification was made by digesting pAX 131 with EcoR I and Xba I. An insert that replaced the elements removed by such digestion was generated using overlapping oligonucleotides with the following changes: conversion of the Sac I site to a new Xba I site (single underline in primer sequences below), conversion of the original Xba I site to a Not I site (double underline in the primer sequences below), and ending the insert with a Spe I overhang which is compatible with the vector's Xba I digest generated overhang. Ligation of the EcoR I / Spe I insert into the EcoR I / Xba I cut vector resulted in an Spe I / Xba I hybrid which will no longer cut with either Spe I or Xba I at that site. The sequence of the oligos used were: "EcoSpe" 5' AA TTC AAG GAG TTA ATT ATG AAA AAA ACC GCG ATT GCG ATT GCG GTG GCG CTG GCG GGC TTT GCG ACC GTG GCC CAG GCG GCC TCT AGA ATC TGC GGC CGC a 3'(SEQ. ID. NO: 107), and "SpeEco" 5' ct agt GCG GCC GCA GAT TCT AGA GGC CGC CTG GGC CAC GGT CGC AAA GCC CGC CAG CGC CAC CGC AAT CGC AAT CGC GGT TTT TTT CAT AAT TAA CTC CTT G 3' (SEQ. ID. NO: 108).

The intermediate vector created was pAX131Xba/Not. The human kappa constant region was inserted between the Xba I and Not I sites generating pAX131-kappa (see Figure 18). The human kappa constant region was PCR amplified from human cDNA using primers that introduced the upstream Xba I site and in the

5   downstream position a TAA stop codon followed by a Not I site. The primers used were CKXba I (5' GGA GTC TAG ATA ACT GTG GCT GCA CCA TCT GTC TTC 3') (SEQ. ID. NO: 109) and CKNotI (5' AGG AGC GGC CGC TTA ACA CTC TCC CCT GTT GAA GCT C 3') (SEQ. ID. NO: 110).

The light chain cloning modifications incorporated into pAX131-kappa were

10   shuttled into the pRL5 vector (modified to have its Not I site removed as described above) by moving the EcoR I to Xho I fragment. See Figures 19 and 20. This vector was designated as pRL5-kappa. (See Figures 21A-I.)

<u>EXAMPLE 7</u>

<u>(core H-CDR2 library)</u>

15   An additional HC CDR2 library was constructed where a core sequence of the TPO mimetic peptide (GPTLRQWL) (SEQ. ID. NO: 112) flanked by a single random amino acid on each side was inserted into the heavy chain partially replacing the CDR2 (GXGPTLRQWLXYAQKFQG) (SEQ. ID. NO: 113). The construction of the heavy chain partial CDR2 replacement library was also carried out as previously

20   described for the heavy chain CDR2 library with the exception that the specific primer 8HCR2anti0 (5'CAGCCACTGGCGCAGGGTTGGGCCMNNCCCTCCCATCCACTCAAGCCC-3') (SEQ. ID. NO: 114) which annealed at the end of the heavy chain FR2 and the primer 8HCR2code(5'GGCCCAACCCTGCGCCAGTGGCTGNNKTACGCACAGAAATTCCAG

25   GGCAGAGTCACCATT-3') (SEQ. ID. NO: 115) which annealed to the beginning of the heavy chain FR3 were used in place of the previously described HR2 cMpl primers. The heavy chain partial CDR2 replacement library was panned four rounds on 293 EBNA cells transfected with the cMpl-R as described previously. These clones can now be screened for positive binding clones by FACs analysis as previously

30   described.

It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended herein.